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A Genome Wide Search for Alcoholism Susceptibility Genes

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Alcoholism is currently one of the most serious public health problems in the US. Lifetime prevalence rates are relatively high with one in five men and one in 12 women meeting criteria for this condition. Identification of genetic loci conferring an increased susceptibility to developing alcohol dependence could strengthen prevention efforts by informing individuals of their risk before abusive drinking ensues. Families identified through a double proband methodology have provided an exceptional opportunity for gene-finding because of the increased recurrence risks seen in these sibships. A total of 360 markers for 22 autosomes were spaced at an average distance of 9.4 cM and genotyping performed for 330 members of these multiplex families. Extensive clinical data, personality variation, and event-related potential characteristics were available for reducing heterogeneity and detecting robust linkage signals. Multipoint linkage analysis using different analytic strategies give strong support for loci on chromosomes 1, 2, 6, 7, 10, 12, 14, 16, and 17.

KEY WORDS: alcoholism; alcohol dependence; genetic susceptibility; linkage analysis

INTRODUCTION

Abuse of alcohol and other substances is one of the most serious public health problems in the United States [Hanson and Li, 2003]. Alcohol dependence (alcoholism) is defined by the APA (Diagnostic and Statistical Manual) as including neglect of responsibilities, persistent drinking in spite of recognition that it is causing harm, inability to reduce intake, and use of alcohol to prevent withdrawal symptoms. Using this definition, alcohol dependence is a common condition with lifetime prevalence rates at 20.1% for men and 8.2% for women [National Comorbidity Survey—Kessler et al., 1997].

Alcohol dependence (alcoholism) contributes to a variety of health problems including increased risk for cancers of the head and neck, hepatocellular carcinoma, cardiomyopathy, hypertension, coronary artery disease and stroke, liver disease including cirrhosis, fetal alcohol effects including Fetal Alcohol Syndrome, increased violence and accidents including those resulting from drunk driving [Hill, 1995a; Lieber, 1995]. Although lifetime prevalence in males appears to be higher than in females, the health and social consequences of alcohol use are quite substantial for women world wide [Hill, 1994, 1995a]. The health costs have recently been estimated to be 184.6 billion, an increase of 25% since 1992 [Harwood, 2000]. The social, physical, and economic costs of excessive use of alcohol are quite significant across both genders and across all ages.

Alcoholism tends to run in families with risk to first-degree relatives exceeding the population prevalence twofold to threefold in most studies [Cotton, 1979]. Evidence that genetic factors mediate this tendency for alcohol dependence to aggregate in families comes from several sources. Some evidence comes from twin studies which tend to show greater concordance for alcohol dependence in MZ than in DZ twins [Kendler et al., 1992; McGue et al., 1992], providing estimates of heritability in the range of 0.54–0.58 in males [Prescott, 2001]. Interestingly, the substantial heritability seen for alcohol dependence has been shown to be consistent across birth cohorts from the early 1900s to present and across different societies [Heath et al., 1997; Kendler et al., 1997]. Segregation analyses have been carried out to test for Mendelian modes of inheritance and to detect the presence of loci of major effect [Aston and Hill, 1990; Yuan et al., 1996]. Mendialian transmission was not supported though evidence was found indicating that the underlying liability to develop alcoholism was, in part controlled by a major genetic effect.

Complex diseases such as diabetes, obesity, major depressive disorder, and alcoholism are influenced by multiple genes, often interacting with environmental conditions to produce the unwanted malady, providing difficult challenges for replicable linkage findings [Allison et al., 1998]. There is abundant evidence that alcohol dependence is a clinically heterogeneous disorder, varying in its age of onset, prevalence by gender, developmental course, and severity [Sher and Trull, 1994; Hill, 1995b, 2000; Finn et al., 1997; Hill and Yuan, 1999]. As in all studies of complex diseases, defining phenotypes that accurately reflect the underlying genetic susceptibility is critical for uncovering susceptibility genes. One of the ways this has been handled in genetic studies is to vary the "bandwidth" of the phenotype, observing changes in the evidence for linkage as one decreases or increases the breadth of the phenotype. One example of how this strategy can be beneficial in finding pleiotropic effects of genetic loci comes from the report by Nurnberger et al. [2001] in which evidence for a locus on chromosome 1 that influences both alcoholism and affective disorder was found using a variable width phenotype.

Another potential method for defining phenotypes that reflect the underlying genetic susceptibility is through the incorporation of covariates thought to be disease relevant, thereby reducing locus heterogeneity. Olson [1999] has developed a conditional-logistic parameterization that generalizes to include several features including the use of covariates. Use of covariates in linkage analyses has the potential for reducing locus heterogeneity making it possible to find linkage signals that may be missed due to heterogeneity within the sample studied. Heterogeneity can weaken or even eliminate evidence for linkage when it is present. The model developed by Olson [1999] has now been successfully applied to a number of

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disorders including prostate cancer [Goddard et al., 2001] and late onset Alzheimer's disease [Olson et al., 2002]. For prostate cancer, it has been shown that incorporation of disease relevant covariates in the analysis of the disorder can shift the evidence from no linkage to significant evidence using such covariates. Similarly, for Alzheimers, a region on chromosome 20 that gave no evidence for linkage without covariates showed greater evidence for linkage with the addition of each disease relevant allele, apolipoprotein E2 and E3, and also by including age of onset so that the three variables resulted in a LOD score of 4.11 ($P = 0.0005$). Comparison of LOD scores resulting from selectively adding covariates is essentially a test of locus heterogeneity allowing the user to determine the importance of a given variable to the overall LOD score result. To our knowledge, these methods have not previously been applied to the alcoholism phenotype.

Use of covariates for investigation of alcohol dependence is facilitated by the existence of quantitative traits that appear to be disease-relevant. These include the P300 component of the event-related potential and particular personality variants (low control or impulsivity). P300 amplitude appears to be related to alcoholism risk based on reports from multiple laboratories. P300 shows moderate heritability and considerable genetic variance using MZ and DZ twin pairs, particularly in males. A few published manuscripts have suggested the possibility that components of the ERP wave, most notably the P300 component, may show linkage to specific chromosomal regions in samples selected for the presence of alcoholism [Porjesz et al., 1996; Begleiter et al., 1998; Porjesz et al., 1998; Williams et al., 1999; Almasy et al., 2001; Hesselbrock et al., 2001]. This endophenotype has been shown to have substantial heritability, a condition needed for adequate testing of the endophenotypic influence [Gottesman and Shields, 1972].

Also, personality variants are quantitative traits that can readily be incorporated in linkage analyses. Personality variation has also been implicated in the diathesis for alcohol dependence. Several characteristics have been suggested including negative affectivity and neuroticism [see Sher and Trull, 1994 for review], alienation [Hill et al., 1990b], and disinhibition/impulsivity [Sher et al., 1991] in offspring of alcoholics. We have found higher scores on the extraversion scale of the Eysenck Personality Inventory (EPI) predicts adolescent age of onset to begin regular drinking among high-risk offspring [Hill and Yuan, 1999; Hill et al., 2000b]. Adult alcoholics score lower on MPQ constraint and control [Hill et al., 1990b]. Those scoring low on this trait would tend to be more impulsive and less prone to careful planning of their actions, a characteristic shared with individuals who are more liking to be sensation-seeking, a trait that has a well-known association with risk for alcohol and drug dependence.

The present analysis focused on identification of possible genetic susceptibility loci using a dichotomous alcoholism phenotype that reflected the presence or absence of alcoholism by Feighner criteria (also they met DSM-III criteria for alcohol dependence). In order to reduce locus heterogeneity, covariates thought to have relevance to the alcoholism phenotype were incorporated in the analyses.

SUBJECTS AND METHODS

Ascertainment and Assessment of Probands and Relatives

Probands were recruited from treatment facilities in the Pittsburgh area. The majority of probands were in inpatient programs at the time of ascertainment. Probands were selected if a pair of same sexed adult siblings with an alcoholism diagnosis were present in the family. Two ongoing awards from NIAAA supported the identification of male alcoholic pairs (Cognitive and Personality Factors in Relatives of Alcoholics Family Study [CPFFS]) and the identification of female alcoholic pairs (Biological Risk Factors in Female Alcoholics Family Study [BRFFS]). Starting in 1984, ascertainment of the male alcoholic pairs was performed to study the personality and event-related potential characteristics of these pairs, their siblings and parents. Later, the offspring from these pedigrees were entered into a longitudinal follow-up. Starting in 1990, the BRFFS initiative ascertained pairs of adult alcoholic sisters and their family members to study ERP and personality characteristics of the family members. The studies had identical ascertainment requirements. However, the study of female alcoholic families was able to enlist fewer siblings for complete ERP and personality assessments. Third generation minor offspring are currently being followed in longitudinal initiatives. Starting in 1990 DNA was banked as participants entered the studies. In order to complete DNA collection for all informative individuals willing to participate in the DNA portion of the study, many individuals were recontacted, having originally given their permission to do so, and asked to provide a blood sample.

The result of this strategy is that the majority of probands have three or more siblings who have contributed DNA, consented to a clinical interview, and provided family history. Of those genotyped in the present report, two-thirds also had available personality assessments, complete ERP evaluations, and extensive health and self-report measures taken. Our rationale for having initiated the study through a double proband sampling scheme was based on the observation that restricting family ascertainment to multiplex families increases the likelihood of finding genes related to the disease of interest [Morton and Mi, 1968; Anderson et al., 1986]. This is largely due to the fact that the likelihood of finding a severe form of any particular disorder segregating within families is increased where multiple cases are found. This strategy also increases detection of relevant biological markers (e.g., P300 characteristics) related to the alcoholism phenotype by sampling affected, unaffected and discordant pairs [Steinhauer et al., 1987; Hill et al., 1988].

All proband pairs were personally interviewed in both family studies. In the CPPFS initiative, all adult siblings, and living parents were interviewed in person with each first-degree relative also providing family history on every other member of the family, allowing for verification of self-reported symptoms. The BRFFS initiative conducted personal interviews for all living and cooperative first-degree relatives for whom blood samples were obtained. The interview format was a semi-structured, well-known psychiatric diagnostic instrument (Diagnostic Interview Schedule [DIS]) with known psychometric characteristics. The DIS was developed in St. Louis and assessed for both reliability and validity [Helzer et al., 1985]. A computer algorithm has been developed by the authors that allows for determining if criteria for particular disorders by each nosologic system are present. Use of this instrument makes it possible to make diagnoses of alcohol dependence, alcoholism, and alcohol abuse by Feighner Criteria [Feighner et al., 1972], DSM-III and IIIR [American Psychiatric Association, 1982]. Because the majority of individuals were assessed before the release of DSM-IV, no attempt was made to re-diagnose the sample to conform to currently prevailing nomenclature. However, to insure the highest quality data possible, the DIS interview data was supplemented by a second interview with a second clinician using a more open-ended format designed to cover major areas of psychopathology routinely covered by clinicians in screening diagnostic interviews. Using the DIS information, the second clinician's information and family history report of all other participating relatives, a "best estimate" diagnosis was made using Feighner criteria. Although symptoms were retained in computer files, which

would allow for quantifying the alcoholism phenotype, the present report is based on the dichotomous phenotype, alcoholism (alcohol dependence).

It is estimated that more than 5,000 families have been screened through an available proband to net the cooperative families upon which the present report is based. The large number of families screened in treatment centers was the result of the need to find "double proband" families where minimal comorbidity (Table I) was present. Families who lived in the Pittsburgh area were given preference for entry into the study so that it would be possible to conduct in-person interviews and event-related potential testing in our laboratory. The present report is based on 65 families and 330 genotyped individuals, which resulted in 418 sib pairs (201 affected/affected pairs, 172 unaffected/affected pairs, and 45 unaffected/unaffected pairs. The sibship size of the families is large with 80% of the families having three or more siblings present (24.6% had three sibs, 24.6% had four, 20% had five, and 10.7 had six or more siblings). One or both parents have been genotyped in 86% of the families (49.2% had one parent genotyped, 36.9% had both). An average of 5.1 individuals per family have been genotyped. Although the overall minority rate in our series of families is 13%, for the genotyped sample 2% were minority. This is due to the fact that obtaining blood samples from our minority families proved to be more difficult.

Because the selection criteria required the presence of a same-sexed proband pair and excluded families with particular psychiatric history, only one family in 100 could be selected and studied. Families were excluded if the proband or any first-degree relative were considered to be "primary" for drug dependence (preceded alcoholism onset by at least 1 year), where 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.

Genotyping

Samples. Blood was drawn from the present set of 330 adult family members with one aliquot being used to extract DNA from whole blood and the second aliquot prepared for EBV tranformation and cryopreservation. DNA samples used in this mapping effort were amplified with the ABI Linkage Marker Set Version 2 (LMSV2) primers. A total of 360 markers for 22 autosomes were spaced at an average distance of 9.4 cM. Two intervals were greater than 20 cM.

Genotype determination. PCR products were analyzed on a Perkin-Elmer Model 377 Automated Sequencer and electrophoresis data transferred to a Power Mac G3 and tracked as batches using GENESCAN 3.1.2. This allowed for manually tracking of each gel before analysis. Each gel included two CEPH DNA (1347-02) samples to control for gel to gel allele-calling variability. Also included on each gel were allelic ladders that were created by pooling 90 DNA aliquots from the sample population. Within the same lanes as each sample were fluorescent size markers (GeneScan 400HD, Applied Biosystems, Foster City, CA) used to assign integer values (bins) to each peak in the allelic ladder. These bins were then used to assign allele sizes to the sample peaks.

Allele calling was first performed using TrueAllele™ automated allele-calling software (Cybergenetics Corp., Pittsburgh, PA). TrueAllele was developed to address one of the main limitations in high-throughput microsatellite genotyping, namely, the manual editing of allele calls. TrueAllele uses deconvolution and quantitation algorithms to assign allele sizes to signal peaks [Perlin et al., 1995]. Although substantial progress has been made in eliminating the need to edit allele calls, the possible loss of information inherent in reliance on a computer algorithm was unknown. Therefore, from the inception of the genome scan, it was determined that maximal error trapping would be possible if two independent readers reviewed the automated allele calls. In order to fully test laboratory genotyping accuracy using this computer algorithm/two reader method, readers were blind to family membership status.

Data editing. The GeneScan gel files were converted to TrueAllele™ Data Disk templates by an experienced researcher at Cybergenetics Corp. The templates analyzed contained the required information needed to define the complete range of sizes of all possible alleles for each particular marker. The TrueAllele software tracks each gel lane and measures the intensity and size of each peak profile. Using size standard and allelic ladder data, TrueAllele then assigns integer values to each measured peak to generate a genotype. A quality value score for each genotype is automatically determined using criteria that define the parameters of an optimal allele peak [Pálsson et al., 1999]. Genotypes that failed one or more of the criteria were assigned "quality" value scores of zero. TrueAllele assigned genotypes of increasing quality with progressively higher quality value scores. This allows the user to choose some arbitrary cut-off value to use in reading electropherograms. In the present study, all genotypes and their associated electropherograms were examined. The assigned genotypes and corresponding quality scores were stored as a Results file in Excel spreadsheet format. The Results files and TrueAllele™ peak profiles were then copied and transferred to an independent reader.

Genotype determination. The readers independently reviewed the peak profiles of all genotypes. A genotype was assigned for all genotypes that could be interpreted and were

TABLE I. Lifetime Prevalence of Comorbid Disorders Percentage of Cases in Sample Genotyped

	Male (N = 168)			Female (N = 162)		
	Alcoholic primary (N = 118)	Alcoholic secondary (N = 3)	Not alcoholic (N = 47)	Alcoholic primary (N = 67)	Alcoholic secondary (N = 13)	Not alcoholic (N = 82)
Major depression	0.0	0.0	2.1	4.5	38.5	16.3[b]
Antisocial personality	21.4	0.0	6.4	22.4	7.7	1.3
Drug dependence	23.9[a]	66.6	0.0	40.3[a]	76.9[d]	2.6[c]
Anxiety disorders	1.7	0.0	0.0	3.0	7.7	3.9
Any comorbidity	36.7	66.6	8.5	56.7	100.0	20.0

[a]Secondary drug dependence (note proband alcoholics were selected to be primary for alcohol dependence). Primary was defined by the age of onset of the disorder. A disorder had to precede another disorder by at least 1 year to be considered primary.
[b]All major depressive disorders in these relatives was the primary disorder. As noted, none were alcoholic and none were dependent on drugs.
[c]This percentage is based on two individuals who met criteria for primary drug dependence and were the relatives of the proband. None were probands.
[d]This percentage is based on ten cases who either met criteria for primary drug dependence (four cases) or met criteria for secondary drug dependence (six cases). None were probands.

stored in an Excel file for each reader allowing comparison of their results. All cases in which the assigned genotype differed between readers were noted in a "discordant" column of the Excel file allowing the readers to further discuss their calls. Review of the discordant calls was done with both readers present. A consensus call was required for each genotype. If a consensus could not be reached or both judged the data to be "missing," the genotype was entered as a "0" value for both alleles. After calculating the proportion of loss, those discordances that remained unresolved were considered as genotype losses and removed from the database before running PEDCHECK [O'Connell and Weeks, 1998]. Any genotypes that were found to be inconsistent with Mendelian inheritance were removed before performing linkage analyses. Use of these error trapping methods has resulted in good genotyping accuracy (0.25%) for the entire genome scan with a low rate of "missing" calls (1.8%) [Hill et al., unpublished].

Personality variation. All adult participants in our family study were administered the Multidimensional Personality Questionnaire [MPQ, Tellegen, 1982]. To minimize type 1 error, the present analysis utilized only one scale, the constraint scale. The assessment of risk taking has relied extensively on self-report instruments that measure constructs such as sensation seeking [Zuckerman et al., 1978], venturesomeness [Eysenck et al., 1985], impulsivity [Barratt, 1985], and behavioral constraint [Tellegen and Waller, 1982]. All of these constructs appear to overlap with risk taking behavior, a tendency which has been linked to deleterious health consequences including alcohol and drug dependence [DiClemente et al., 1995]. The constraint scale of the MPQ is a higher order scale consisting of Control, Harm Avoidance, and Traditionalism scales, which are three of the 11 primary scales of the MPQ. This instrument has good test–retest reliability with correlations ranging between 0.82 and 0.90 [Tellegen and Waller, 1982]. Further, phenotypic, additive genetic, and nonshared environmental correlations have been computed for 2,490 twin pairs among the 11 primary scales revealing three broad higher-order components by factor analysis that can be interpreted as negative emotionality, positive emotionality and constraint which map well to the etiological structure of personality [Krueger, 2000].

P300 assessment. ERP assessments are routinely performed in our laboratory using two oddball tasks: an auditory task (Choice Reaction Time task and Counting Task) as well as a Visual Discrimination task. The EEG is recorded from scalp locations Fz, Cz, Pz, P3, P4, and Oz using a linked ear reference and an EOG artifact channel, digitized at 125 Hz for 1,200 msec beginning 200 msec prior to stimulus onset. Grass Neurodata amplifiers are set with a bandpass of 0.01–30 Hz. Ground is placed at the forehead, and impedances are less than 5 KΩ. Trials are excluded when the EOG exceeds approximately 75 μV. In all paradigms, standard signal averaging procedures were employed to obtain the average ERP waveform from the single trials. P300 component values were measured by a computer algorithm that identifies the largest peak within a specific latency window and is verified by two independent raters. Amplitude was defined from the mean of the prestimulus baseline voltage to the peak.

Auditory ERPs are elicited with a 800 Hz "low" tone and a 1,500 Hz "high" tone (40 msec duration—3 sec interstimulus interval—70 dBA intensity) in a modified oddball paradigm. Briefly, high-pitched tones occur 25% of the time. Thus, three conditions could be identified by their conditional sequential probability; an unpredictable rare high tone ($P = 0.33$), an unpredictable low tone ($P = 0.67$), and a predictable low tone ($P = 1.0$) which occurred after each high tone. The condition with the probability of 1.0 was the "certainty" condition due to prerecording instruction to subjects that two high-pitched tones could not occur in sequence. Subjects are asked to press a button corresponding to the tone heard while seated in a sound-attenuated chamber. To minimize type 1 error, the present analysis was based on only a single auditory task (Counting Task), one electrode (Cz), and the 0.33 probability condition.

Statistical methods. After the data set was checked for Mendelian inheritance of the marker alleles, the USERM13 option of the MENDEL suite of linkage programs [Boehnke, 1991] was used to calculate maximum likelihood estimates of marker allele frequencies. Results are presented for multipoint linkage analyses completed using data for adult full siblings and parents using version 4.2 of SIBPAL [SAGE, 2002] from the SAGE package (Statistical Analysis for Genetic Epidemiology). SIBPAL estimates the proportion of alleles inherited identical by descent (IBD) for each marker for all possible affected sib pairs n (n – 1)/2 for n affected sibs, and compares this to the null hypothesis (0.50 sharing) using a *t*-test [Blackwelder and Elston, 1985]. Decreased sharing among discordant pairs and increased sharing among unaffected pairs was also estimated. When parental genotypes are missing, allele frequency estimates provide a weighted estimate of IBD. It should be noted that DNA was available for one or both parents in 86% of the families included. To evaluate evidence for linkage, SIBPAL was used to evaluate the mean IBD sharing in affected, discordant, and unaffected sib pairs [Haseman and Elston, 1972]. IBD sharing by sibpair status along with nominal *P* values may also be seen in Table II.

Greenwood and Bull [1997, 1999] and Olson [1999] have shown that logistic regression models can easily allow covariates to be included in an affected sib pair analysis (ASP) and that inclusion of important covariates can substantially

TABLE II. Nominally Significant Loci in SIBPAL Multipoint Analyses

	IBD sharing calculations from SIBPAL		
Markers	P(IBD) UU	P(IBD) AU	P(IBD) AA
D1S230	0.48	0.54	0.56**
D1S2841	0.47	0.49	0.55*
D2S319	0.47	0.53	0.54*
D3S1297	0.48	0.51	0.54*
D3S1580	0.51	0.49	0.56**
D3S1601	0.53	0.49	0.57***
D3S1311	0.50	0.49	0.56**
D5S436	0.44	0.51	0.54*
D6S1574	0.56	0.54	0.58***
D6S309	0.56	0.51	0.58**
D6S470	0.52	0.51	0.57**
D6S462	0.46	0.49	0.55*
D7S517	0.57*	0.49	0.55**
D7S486	0.47	0.51	0.56**
D7S636	0.47	0.50	0.54*
D8S277	0.52	0.50	0.54*
D9S288	0.44	0.50	0.56**
D9S158	0.53	0.52	0.53*
D13S175	0.50	0.50	0.56**
D13S217	0.43	0.44*	0.55*
D13S1265	0.49	0.49	0.55*
D17S921	0.52	0.45*	0.55*
D17S1857	0.50	0.45*	0.56**
D17S798	0.51	0.46*	0.55**
D17S1868	0.51	0.45*	0.57**

P(IBD) scores shown in the table are the estimates of the average proportion of alleles shared identical by descent (IBD) for 43 concordantly unaffected (UU) pairs, 171 discordant (AU) pairs, and 201 concordantly affected (AA) pairs using SIBPAL. Bolded markers are significant at LOD > 3.0 ($P = 0.006$) in the three covariate LODPAL model.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

increase the power to detect linkage. The method developed by Olson [1999] is available in SAGE (LODPAL). All LODPAL analyses were planned in advance in order to reduce the likelihood of type I error. Because gender is an important variable affecting the prevalence of alcohol dependence, the first step was to include gender along with age in the analyses of the 22 autosomes. Age is important because of the clear effect which age at interview has on diagnostic status. Unaffected individuals who are young at the time of diagnosis can become affected at a later point in time.

RESULTS

Age and Gender as Covariates

Figure 1 illustrates the results of performing linkage analysis with LODPAL, a conditional logistic regression technique that allows the user to incorporate covariates into the linkage analysis using a dichotomous phenotype. Results are plotted for the three parameter model which includes the baseline, alcoholism diagnosis by DSM-III, and Feighner et al. [1972] criteria [yes or no] and using age and gender as covariates. As may be seen in Table III, five chromosomal regions on chromosomes 5, 6, 17, and 20 revealed LOD scores ≥ 3.0 ($P < 0.002$), using the two covariate model (age and gender). The impact of including age and gender in the model relative to the results obtained using the baseline alcoholism diagnosis can be seen in Table III. As may be noted, the addition of age and gender as covariates in the model was significant at $P < 0.01$ for 15 markers indicating that addition of these variables reduced heterogeneity. The next step was to determine if adding either P300 amplitude or constraint in our model would reduce heterogeneity further.

Age, Gender, and P300 Amplitude (Counting Task at Cz Electrode)

A four-parameter model was evaluated using LODPAL by including amplitude of the P300 signal along with age and gender and the alcoholism diagnosis. As may be seen in Table III, seven markers in regions on chromosomes 5, 6, 12, 14, and 17 had LOD scores greater than 4.0 ($P = 0.001$). Results are depicted in Figure 2 for these regions, and for chromosome 7 which reached a LOD score of 3.54 for marker D7S517. (The chromosome 5 markers showing LODS > 4.0 are not depicted because of inconsistent performance in the alternate three covariate analyses.) As may be seen in Figure 2, two peaks with LOD values >4.0 were found on chromosome 6, the maximum value being seen at marker D6S1574 with a LOD score of 5.86 ($P = 0.000013$). For chromosome 7, the maximum LOD score of 3.54 was significant at $P = 0.0018$. Three regions on chromosome 12 showed LOD scores greater than 3.0 in the three covariate model that included P300. A maximum LOD score of 4.35 ($P = 0.0003$) was obtained at D12S346. For chromosome 14, marker D14S258 gave a LOD score of 4.64 ($P = 0.00018$). Two peaks greater than 3.0 were seen for chromosome 17. The broader peak at about 50 cM which included marker D17S1857 gave a LOD score of 4.32 ($P = 0.00035$), with the adjacent marker D17S798 having a LOD score of 3.68 ($P = 0.0014$). The other peak was at marker D17S928 with a LOD score of 4.00 ($P = 0.0007$). The impact of including the P300 amplitude variable in the analysis was evaluated by subtracting the LOD value for the two covariate model from the three covariate model (Table III). As may be seen, P300 contributed significantly ($P = 0.01$) as a covariate at six markers.

Age, Gender, and Constraint

A four-parameter model was evaluated by including measurement of constraint along with age, gender, and alcoholism diagnosis. Using this model, 14 markers in chromosomal regions on chromosomes 1, 2, 6, 7, 10, 12, 14, 16, and 17 showed LOD scores ≥ 3.0 (see Table III). Figure 2 displays results for constraint for chromosomal regions on 1, 2, 10, and 16, regions where the maximum LOD score at a particular marker was obtained using the three covariate model that included constraint. In contrast, maximal LOD values in the three-covariate model that included P300 were seen for regions on chromosomes 6, 7, 12, 14, and 17.

Where maximal LOD values were obtained in the model that included constraint, we note that chromosome 1 had a maximal LOD score of 3.46 ($P = 0.0022$) at marker D1S196, with a LOD value of 3.45 ($P = 0.0022$) at the adjacent marker D1S2878

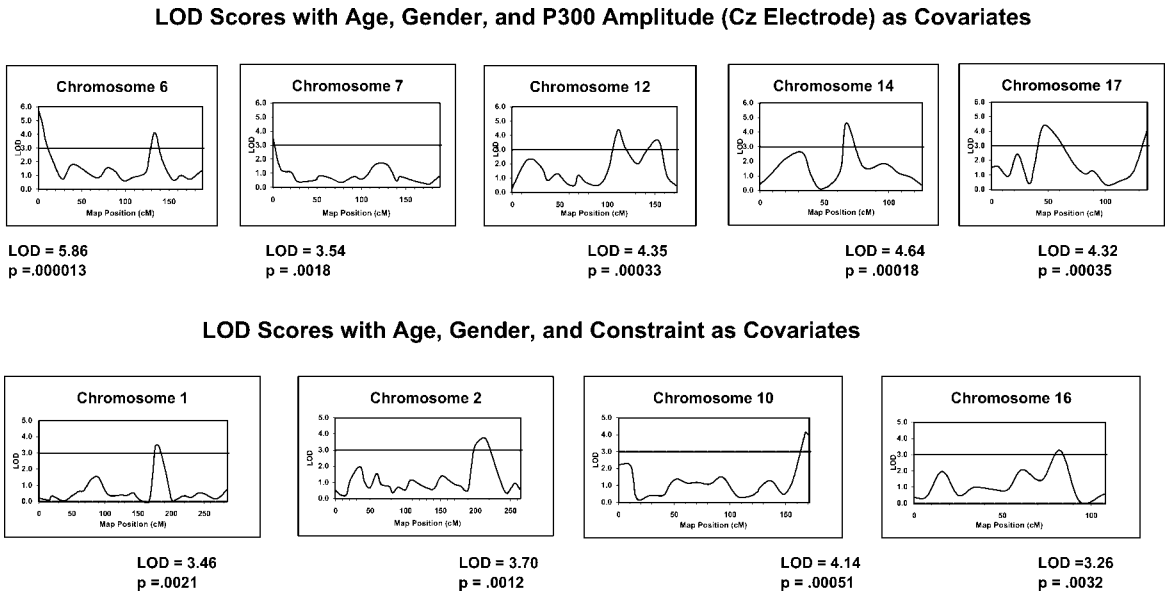

Fig. 1. LOD scores for the genome scan for the binary alcoholism phenotype with age and gender as covariates. Horizontal lines correspond to a LOD score of 3.0 ($P = 0.0006$).

TABLE III. LOD Values at Baseline Using the Binary Alcoholism Phenotype Along With Age, Gender, and P300 or Constraint

	Alcoholism		Alcoholism gender and age		Alcoholism gender age and P300		Alcoholism gender age and constraint		Effect of adding age and gender		Effect of adding P300		Effect of adding constraint	
Marker	LOD	*P* value	LOD	*P* value	LOD	*P* value	LOD	*P* value	LOD	*P* value	LOD	*P* value	LOD	*P* value
D1S2878	0.14		1.24		1.25		**3.45**	0.002	1.10		0.01		2.21	0.001
D1S196	0.07		1.12		1.09		**3.46**	0.002	1.05		−0.03		2.34	0.001
D2S117	0.05		**2.05**	0.017	2.15	0.031	**3.07**	0.005	2.00	0.010	0.10		1.02	0.030
D2S325	0.00		0.92		1.08		**3.70**	0.001	0.92		0.16		2.78	0.000
D2S2382	0.00		1.38		1.56		**3.68**	0.001	1.38	0.042	0.18		2.30	0.001
D5S647	0.17		**2.14**	0.014	2.14	0.031	1.90	0.050	1.96	0.011	0.01		−0.24	
D5S424	0.00		**2.62**	0.005	2.40	0.019	2.77	0.009	2.62	0.002	−0.22		0.15	
D5S644	0.03		**3.54**	0.001	**4.07**	0.001	0.00		3.52	0.000	0.53		−3.54	
D5S433	0.07		**2.87**	0.003	2.88	0.007	0.00		2.80	0.002	0.01		−2.57	
D6S1574	2.17	0.001	**4.25**	0.000	**5.86**	0.000	**5.01**	0.000	2.08	0.008	1.61	0.007	0.76	
D6S309	1.81	0.002	**3.98**	0.000	**4.65**	0.000	**4.02**	0.001	2.17	0.007	0.66		0.03	
D6S470	1.47	0.005	**2.35**	0.009	**3.23**	0.003	2.27	0.024	0.88		0.87	0.045	−0.08	
D6S308	0.04		**2.86**	0.003	**4.10**	0.001	**4.51**	0.000	2.82	0.002	1.24	0.017	1.65	0.006
D7S517	1.70	0.003	**2.65**	0.005	**3.54**	0.002	2.37	0.020	0.95		0.89	0.043	−0.28	
D8S550	0.92	0.020	**2.29**	0.010	2.99	0.006	2.30	0.023	1.37	0.043	0.70		0.01	
D10S249	0.00		**2.11**	0.015	**3.03**	0.005	2.24	0.026	2.11	0.008	0.92	0.039	0.14	
D10S1651	0.08		0.34		0.37		**4.14**	0.001	0.26		0.03		3.80	0.000
D10S212	0.01		0.90		1.74		**3.99**	0.001	0.89		0.84	0.049	3.09	0.000
D12S99	0.26		**2.32**	0.009	2.13	0.032	2.44	0.017	2.06	0.009	−0.20		0.11	
D12S346	0.01		**2.71**	0.004	**4.35**	0.000	**3.25**	0.003	2.71	0.002	1.64	0.006	0.54	
D12S78	0.00		1.17		**3.18**	0.004	1.57		1.17		2.01	0.002	0.41	
D12S324	0.74	0.032	1.77	0.030	**3.64**	0.001	0.00		1.03		1.87	0.003	−1.77	
D13S171	0.24		**2.23**	0.011	1.99	0.042	1.66		1.99	0.010	−0.24		−0.57	
D13S218	0.50		**2.19**	0.012	1.96	0.045	1.53		1.68	0.021	−0.23		−0.65	
D13S263	0.19		**2.14**	0.014	2.96	0.006	1.55		1.94	0.011	0.82	0.052	−0.59	
D13S1265	0.65	0.042	**2.10**	0.015	2.42	0.018	1.58		1.45		0.32		−0.52	
D14S70	0.00		**2.30**	0.010	2.59	0.013	2.56	0.014	2.30	0.005	0.29		0.26	
D14S258	0.10		1.52	0.051	**4.64**	0.000	**3.51**	0.002	1.42	0.038	3.12	0.000	1.99	0.002
D16S516	0.00		0.63		0.73		**3.26**	0.003	0.63		0.10		2.63	0.001
D17S1852	0.74	0.033	**2.11**	0.015	2.44	0.017	**3.26**	0.003	1.37	0.043	0.33		1.16	0.021
D17S1857	1.39	0.006	**2.11**	0.015	**4.32**	0.000	2.40	0.019	0.71		2.21	0.001	0.29	
D17S798	1.64	0.003	**2.85**	0.003	**3.68**	0.001	**3.08**	0.005	1.21		0.83	0.051	0.22	
D17S928	0.01		**3.18**	0.001	**4.00**	0.001	**3.66**	0.001	3.17	0.001	0.82	0.052	0.48	
D18S53	0.30		**2.95**	0.002	**3.22**	0.004	2.13	0.032	2.65	0.002	0.27		−0.82	
D20S119	0.00		**4.51**	0.000	0.00		0.00		4.51	0.000	−4.51		−4.51	

Markers are included in the table if the baseline LOD value with age and gender was >2.0, or >3.0 in a three covariate model (bold).

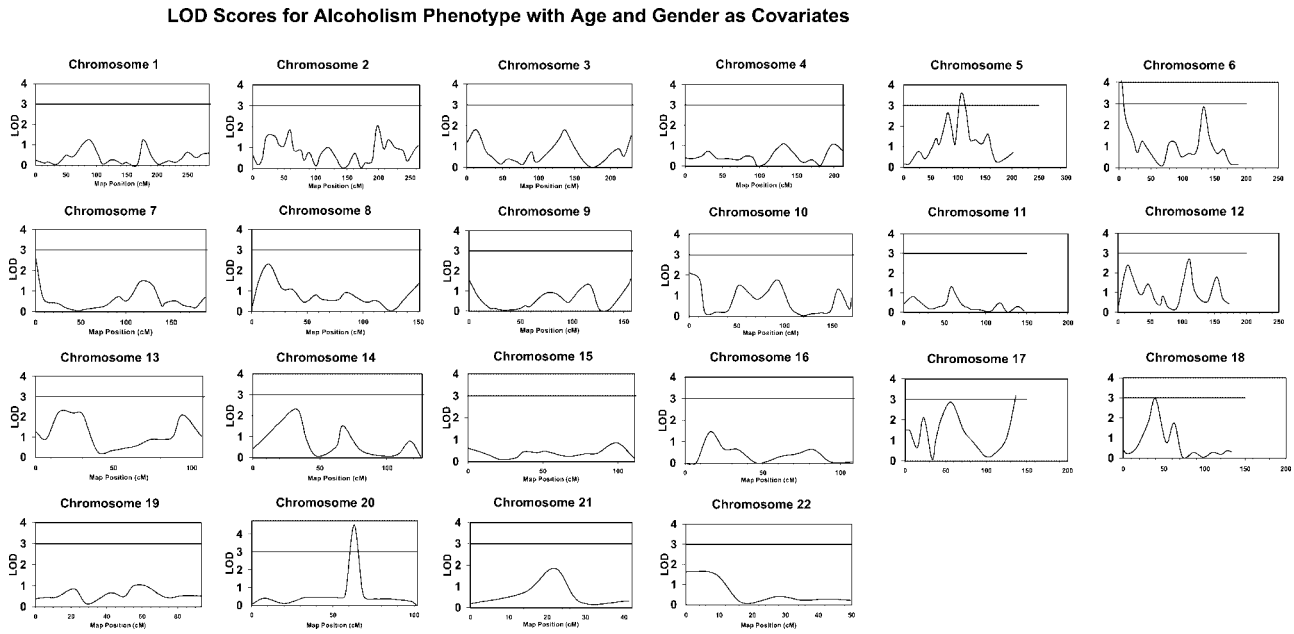

Fig. 2. Chromosomal regions are depicted for three covariate models that resulted in maximal LOD scores for a given model that included the binary alcoholism phenotype, age, gender, and either P300 (counting task at Cz electrode) or constraint as covariates. Horizontal lines correspond to a LOD score of 3.0 $P = 0.0006$).

(Fig. 2). Chromosome 2 showed a peak with a LOD score of 3.70 ($P = 0.0013$) at marker D2S325, and at the adjacent marker D2S2382, a LOD score of 3.68 ($P = 0.0014$) was found. A lesser peak was also seen at D2S117 with a LOD of 3.07 ($P = 0.005$). For chromosome 10, a maximal LOD score of 4.14 ($P = 0.0005$) was obtained at marker D10S1651 with a LOD value of 3.99 ($P = 0.0007$) at the adjacent marker D10S212. Chromosome 16 showed a maximal peak at marker D16S516 with a LOD score of 3.26 ($P = 0.0033$). The impact of including the constraint scale variation in the analyses may be seen in Table III.

DISCUSSION

Although two genome-wide studies of alcoholism are underway, one in Australia and one in Ireland, other than the present study, the only other completed study for which there are published results is the Collaborative Study on the Genetics of Alcoholism (COGA).

The present results were based on a genome-wide study of the DSM-III alcoholism phenotype using multipoint statistical methods (LODPAL and SIBPAL) in an effort to uncover genomic regions that might contain alcoholism susceptibility genes. A number of potential candidate genes exist in the 11 regions where relatively robust LOD scores ($P < 0.002$) on nine chromosomes (1, 2, 6, 7, 10, 12, 14, 16, and 17) were found. Two peaks on chromosome 1 were found that are of interest. One peak is near D1S230, a region where the leptin receptor (OBR), and a tyrosine kinase gene are located. A second region which includes markers D1S196 and D1S2878 is within 1 cM of the gene for an aldehyde dehydrogenase (ALDH9), an enzyme that catalyzes gamma aminobutylaldehyde to GABA. The region near D1S230 has previously been reported by the COGA group to give evidence for linkage [Reich et al., 1998; Foroud et al., 2000; Nurnberger et al., 2001; Dick et al., 2002]. In the present study, multipoint linkage analysis showed a LOD value of 3.46 ($P = 0.002$) for marker D1S196 [181.49 cM Marshfield] and a similar LOD value (3.45) at the adjacent marker D1S2878 using a three covariate model that included age, gender, and constraint. This marker is within 20 cM of marker D1S518, a marker reported by Dick et al. [2002] to have a LOD score of 3.30. Also, we find nominal evidence for linkage based on identity by descent (IBD) sharing for affected pairs (0.56, $P < 0.01$) using multipoint SIBPAL analysis for marker D1S230 (95 cM Marshfield).

For chromosome 2, a region that included D2S235 gave a LOD score of 3.70 using a four parameter model that included the constraint scale as one of the covariates. This signal is in a region that contains the *CREB 1* gene, a gene that produces a response-element binding protein that is activated in response to ethanol and other drugs of abuse. This activation is also involved in the expression of genes that are induced rapidly and transiently (e.g., immediate early gene [IEG] expression such as c-fos). Two peaks were seen on chromosome 6, one giving a maximal LOD score of 5.86 at marker D6S1574 and another at D6S308 with a LOD score of 4.51. The peak near D6S1574 was revealed in a four parameter model involving P300 amplitude. This finding is of interest because of the presence of the *NRN1* gene within 1 cM of D6S1574. The *NRN1* gene is a glutamate and neurotropin receptor target gene, which encodes a protein that functions extracellularly to modulate neurite growth. For the region on chromosome 10, we find the maximal LOD score (4.14 using a four-parameter model that included the constraint scale) at D10S1061. This marker is located within 3 cM of the cytochrome P450 (ethanol induced) gene (*CYP2E*). This region has previously been implicated in linkage analyses of the "low response to alcohol" phenotype seen in family history positive individuals in response to acute doses of alcohol (Subjective High Assessment Scale and body sway) [Wilhelmsen et al., 2003].

On chromosome 12, the maximal LOD score of 4.35 was seen at marker D12S346 using a four-parameter model that included P300 amplitude. This marker is within 3 cM of a GABA A receptor (type B3). A region on chromosome 17 was found with a LOD score of 4.32 at marker D17S1857. This region contains the serotonin transporter gene, a gene that has been implicated in anxiety-related traits [Lesch et al., 1996], alcohol dependence [Schuckit et al., 1999], and in greater consumption of alcohol in non-human primates [Heinz et al., 2003].

Additionally, this region contains the *ALDH3* gene, a gene that produces aldehyde dehydrogenase, one of the principal enzymes, along with alcohol dehydrogenase (ADH), that is responsible for the oxidation of ingested ethanol. ALDH3 is strongly expressed in the stomach but virtually undectectable in the normal liver. Variations in ALDH3 may be associated with organ damage related to alcohol ingestion [Yoshida, 1994]. In summary, most of the regions showing robust LOD scores were within a few centomorgans of candidate genes that appear to have relevance to alcoholism susceptibility.

Regions on chromosomes 1, 2, 6, 7, 12, 14, and 16 found in the present data set have been reported to be significant at levels that are equivalent to a LOD score of 2.0 or greater by either COGA investigators or in reports resulting from GAW10. COGA [Reich et al., 1998] initially presented data based on a genome-wide search of 105 families that provided nominal evidence (two-point SIBPAL results) for linkage with alcohol dependence for 24 loci on 12 chromosomes. The multipoint linkage analyses reported by COGA provided some evidence for linkage for regions on chromosomes 1, 2, and 7 and a possible protective locus on chromosome 4. The region on chromosome 1, D1S1588 attained a LOD score of 2.93 and was located at [125.5 cM Marshfield units]. The chromosome 7 locus was maximal at marker D7S1793 with a LOD of 3.49. The chromosome 4 region extended 38 cM near D4S2393 and D4S2361 [69.6 cM] and had a LOD score of 2.50. Results of genotyping a replication sample of 157 COGA families supported the linkage findings for chromosome 1 for marker D1S1588, with the combined sample decreasing to LOD = 2.6 [Foroud et al., 2000]. The linkage reported for chromosome 7 at D7S821 increased in the combined data to LOD = 2.9. For chromosome 2, the peak LOD score for the initial sample was 3.0, at marker D2S379 (Marshfield 85.48 cM), a signal that was not maintained in the replication sample. A new finding for chromosome 3 was identified in the replication sample that reached a LOD score of 3.4 [Foroud et al., 2000]. Curiously, there was no discussion of the chromosome 4 finding noted by Reich et al. [1998] in the Foroud et al. [2000] report.

Reanalysis of the region containing D1S1588 by COGA investigators [Nurnberger et al., 2001; Dick et al., 2002] supported the earlier findings of Reich et al. [1998] and Foroud et al. [2000]. Nurnberger et al. [2001] using phenotypes that included, alternatively, alcoholism with comorbid depression, or alcoholism or depression alone obtained LOD scores of 5.12, 1.52, and 4.66 at marker D1S1588 in initial, replication, and combined data sets, respectively. Dick et al. [2002] using a derived phenotype based on a principal component analysis of DSM-IIIR items and scales from the Tridimensional Personality Questionnaire (TPQ) reported evidence for linkage (LOD = 3.3) to a region that included marker D1S518. Finally, using low level of alcohol response as a phenotype, Schuckit et al. [2001] found support for linkage at D1S1588.

With the release of the COGA data set for Genetic Analysis Workshop 10 (GAW 10) several new analyses were performed that confirmed some of the previous reports of Reich et al. [1998] and Foroud et al. [2000] but also identified new regions of interest. Several of these regions have overlap with the results from the present data set as may be seen in Table IV.

The present data set did not find support for the region on chromosome 4 identified by other investigators [Long et al., 1998; Dick et al., 2002] where the most likely candidate gene in the region is the *GABA alpha 1* receptor. Long et al. [1998] studying a Southwestern American Indian tribe found evidence for linkage to four chromosome 4 markers, with the best evidence being found at D4S3242 at approximately 105 cM with a LOD score of 2.8. Similarly, Dick et al. [2002] reported a LOD score of 2.7 at D4S1544 at 100.1 cM (Marshfield units).

Regions of chromosome 11 have given suggestive evidence for linkage. Two studies have found evidence for linkage in a region containing the D4 receptor polymorphism [Long et al., 1998; Hill et al., 1999a]. Long et al. reported a LOD score of 2.11, $P = 0.00007$ for D11S1984. Genotyping the VNTR polymorphism for D4 using the alcoholism phenotype in combination with presence of one or more physical dependence symptoms gave a nominally significant result for 130 affected sib pairs ($P = 0.04$). This result explained approximately 4% of the variance in outcome.

Of particular interest in the present study was the improvement in LOD scores seen for regions on chromosomes 6, 12, 14, and 17 when P300 amplitude in the CT task was used in analyses involving the peak signal for each chromosome. This may be the result of P300 amplitude having relevance to the etiology of alcohol dependence. As noted by Hauser et al. [2003] inclusion of covariates may increase the power to detect linkage either because the covariate is a "nuisance" factor that is used to remove variability (e.g., gender or age), or alternatively as a confounder in the epidemiologic sense. In this latter view, the covariate may be related both to the outcome of interest and the risk factor in question. In this sense, the covariate may be related to the gene itself so that use of the covariate allows for identification of subsets of families segregating a particular susceptibility allele.

The amplitude of the P300 component of the event-related potential has been shown to be reduced in children/adolescents from high-risk families in comparison to controls [Begleiter et al., 1984; Hill et al., 1990a; Hill and Steinhauer, 1993; Polich et al., 1994]. This finding appears to be most strongly related to age (younger children/adolescents in contrast to adults), gender (boys have lower P300 than girls) and the density of alcoholism cases within the family. Our longitudinal follow-up of the same children at yearly intervals shows that developmental trajectories of P300 amplitude differ between risk groups [Hill et al., 1999c] and variation in trajectory pattern may be associated with risk for developing a childhood or adolescent psychiatric disorder [Hill and Shen, 2002]. An association between P300 amplitude at age 10 and later development of alcohol problems was seen in 18 year olds in our laboratory [Hill et al., 1995c]. Similar results were found by Berman et al. [1993] in youngsters assessed for P300 and evaluated 4 years later for substance dependence. McGue et al. [2001] have found that reduced P300 amplitude is associated with age at first drink, alcohol dependence, and a broad array of disinhibitory behavior including nicotine dependence conduct disorder and antisocial personality disorder, and the MPQ trait of constraint.

P300 amplitude in adulthood has been studied extensively in families of alcoholics [Steinhauer et al., 1987; Hill et al., 1988, 1999d; Almasy et al., 1999]. Moreover, comorbid conditions such as depression appear to influence the expression of P300 amplitude at the Pz (parietal) electrode in adulthood [Hill et al., 1999b]. Nevertheless, there is a tendency for non-alcoholic high-risk relatives of alcoholics to have reduced amplitude of P300 at the Cz electrode [Almasy et al., 1999]. Quantitative trait loci analysis of the COGA data set has suggested possible loci for P300 amplitude on chromosomes 2, 5, 6, and 13 [Begleiter et al., 1998].

P300 amplitude reduction may not be specifically related to an increased risk for alcohol dependence as reduction in amplitude has been found in association with other psychiatric disorders. A family study that recorded auditory event-related potentials from members of 20 multiplex families with schizophrenia found P300 latency delayed and amplitude reduced in schizophrenics in comparison to controls and in about half of the asymptomatic relatives, though not all families were characterized by this pattern [Blackwood et al., 1999]. A segregation analysis using ERP characteristics found P300 latency as a marker for predisposition to schizophrenia [Sham et al., 1994]. One high-risk study of children of schizophrenics

TABLE IV. Chromosomal Regions Identified in the Present Genome-Wide Linkage Analyses With Comparison to Previous Reports

	LODPAL			SIBPAL			Results from COGA data set for each region (regions reported are within 30 cM or less)			
	Marker	LOD score	Map (cM)	Marker	LOD score	Map (cM)	Marker	LOD score	Map (cM)	Author(s)
Chr1	D1S196	3.46[b]	181.49				D1S1595	0.90–1.53	161.05	D'Alfonso [1999]; Sheffield et al. [1999]
	D1S2878	3.45					D1S1660	1.72	212.44	Sun et al. [1999]
							D1S518	3.30	202.19	Dick et al. [2002]
Chr1				D1S230	2.17	95.31	D1S532	2.50	104.23	Peterson et al. [1999]
							D1S2614	2.60	104.79	Foroud et al. [2000]
							D1S1648	5.12	101.48	Nurnberger et al. [2001]
							D1S1588	5.12	125.51	Nurnberger et al. [2001]; Reich et al. [1998]
Chr 2	D2S325	3.70[b]	204.53				D2S434	1.85	215.78	Valdes et al. [1999]
							D2S1371	2.22	215.25	Valdes et al. [1999]
Chr 6	D6S1574	5.86[a]	9.18	D6S1574	3.24	9.18	D6S1006	2.39	26.7	Lin et al. [1999]
Chr 7	D7S517	3.54[a]	7.44	D7S517	2.16	7.44	D7S1795	1.00	36.03	Sheffield et al. [1999]
							D7S1793	3.29	69.56	Reich et al. [1998]
Chr 10	D10S1651	4.14[b]	168.77							
Chr 12	D12S346	4.35[a]	104.65				D12S193	1.60–2.00	104.12	Merette et al. [1999]; Reich et al. [1998]
Chr 14	D14S258	4.64[a]	76.28				D14S302	4.00	107.30	Bergen et al. [1999]
Chr 16	D16S516	3.26[b]	100.39				D16S2624	2.36	87.62	Sheffield et al. [1999]
Chr 17	D17S1857	4.32[a]	43.01	D17S1857	2.32	43.01				
				D17S798	2.27	53.41				
				D17S1868	2.69	64.16				

Regions identified by case control association analysis are not included.
Where *P* values are reported values have been converted to LOD score equivalents for comparison purposes.
[a]P3.
[b]Constraint.

found that significantly lower P300 amplitude in these high-risk offspring was most highly correlated with poorer Global Personality Functioning [Squires-Wheeler et al., 1993].

Whether P300 amplitude in childhood is a specific marker or non-specific for psychiatric disorders of adulthood, it is nevertheless an important heritable biological marker worthy of further pursuit in understanding the etiology of psychiatric disorders. For example, a robust LOD score (7.1) has been found at chromosome 1q42 for a translocation that disrupts brain expressed genes and co-segregates with schizophrenia and affective disorders; the best evidence for linkage being found when recurrent major depression, bipolar disorder, and schizophrenia were all classified as affected [Blackwood et al., 2001].

Also, of special interest is the improvement in LOD scores for regions on chromosomes 1, 2, 10, 14, and 16 when the personality variable, constraint, was added as the third covariate in our analyses. These results indicate that removing the effect of a personality characteristic that reflects the individual's tendency to be impulsive or act without thinking, may reduce heterogeneity sufficiently to allow for identification of genetic susceptibility loci. It has generally been recognized that there are personality dimensions that often accompany susceptibility to alcohol dependence including novelty seeking and impulsivity [Li, 2000], and low levels of constraint [Hill et al., 1990b]. Low levels of the MPQ scale constraint have also been found to account for the relationship between history of suicidal attempts and antisocial deviance and indicating a possible common vulnerability for both [Verona et al., 2001].

We recognize that a relatively large number of potentially significant results were found in this set of pedigrees. Using the suggestion of Lander and Kruglyak [1995] for genome-wide significance levels ($P = 5 \times 10^{-5}$), one of our signals (D6S1574) would meet this requirement. However, several other regions were significant at a LOD score of 3.0 or greater, a P value of 0.01 in a four parameter model. Including covariates in the analysis has the potential for increasing the genome-wide type I error rate due to the increased number of tests made. Also, some discussion has taken place with regard to whether the covariate model proposed by Olson and implemented in LODPAL is sufficiently conservative [Devlin et al., 2002a,b], though equally valid arguments have been offered in defense of the model [Olson, 2002a,b]. Resolution of this issue may need to await extensive simulation studies that would provide empirically derived P values. However, there does appear to be some consensus that inclusion of covariates can increase the power to detect genes for complex traits [Hauser et al., 2003].

Finally, the large number of significant results seen in our data set may have been due to the ascertainment scheme used which required a pair of affected siblings for inclusion of a family and required minimal Axis I comorbidity in family members. Future work with this data set will include looking at regions that are supported by more than one statistical methodology, by further examining regions where reports from other laboratories suggest reasonable LOD values, and by generating empirical P values by conducting simulation studies for promising findings. Also, future work will include finer mapping of several of the regions identified in this genome search with follow-up linkage disequilibrium mapping with single nucleotide polymorphisms (SNPs).

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