Pathway based analysis of genotypes in relation to alcohol dependence

MA Reimers¹, BP Riley²,³,⁴, G Kalsi²,⁴, DA Kertes⁵ and KS Kendler²,³,⁴

¹Department of Biostatistics, Virginia Commonwealth University, Richmond, VA, USA; ²Department of Psychiatry, Virginia Commonwealth University, Richmond, VA, USA; ³Department of Human and Molecular Genetics, Virginia Commonwealth University, Richmond, VA, USA; ⁴Virginia Institute of Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, VA, USA and ⁵Department of Psychology, University of Florida, Gainesville, FL, USA

Correspondence:
Dr MA Reimers, Department of Biostatistics, Virginia Commonwealth University, Room 3014, 730 E Broad St, Richmond, VA 23298-0032, USA.
E-mail:mreimers@vcu.edu

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We introduce a method for detecting variants in several genes of related function with small effect on a phenotype of interest. Our method uses logistic regression to test whether multiple alleles within a functional set have significantly higher than expected predictive value, even though none individually may have strong individual effects. We illustrate this method by testing seven gene sets (including 48 genes), from a study with 1350 single nucleotide polymorphisms in 130 addiction candidate genes studied in a sample of 575 alcohol dependence (AD) cases and 530 controls. We conclude that AD is related to variation in genes participating in Glutamate and γ-amino butyric acid signaling, as has been reported elsewhere, and in stress response pathways, but not with genes in several other systems implicated in other drugs of abuse.

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Introduction

Modern efforts to identify the genetic risk factors for complex psychiatric and drug abuse disorders have focused mostly on genomic regions (in linkage analysis) or individual sequence variants (or associated haplotypes) in or near specific genes (in candidate gene association studies). A few studies¹–⁵ have examined epistatic models of disease etiology; these studies have, typically examined only two markers, loci or linked regions at a time. Recently, genome-wide association studies (GWAS) for psychiatric disorders have appeared.⁶–⁹

In this article, we apply a different and systematic approach towards uncovering the genetic basis of complex diseases—one based on the analysis of sets of genes whose protein products collaborate in specific functions. This may be thought of as in-between a candidate gene study and a GWAS. For the purposes of this paper, we define a functional gene set as one whose protein products are involved in a closely coordinated biological function. Although network approaches are common in the analysis of expression arrays, we are aware of only three previous steps in this direction in linkage or association studies in relation to complex disease.¹⁰–¹² These papers have used methods devised for continuous gene expression data and adapted them to discrete genotype data. By contrast, our approach is grounded in the methodology of statistical genetics extended in a natural manner to gene networks.

We conceptualize the gene set approach as follows. Consider a particular set of genes that produce proteins that act in a pathway to produce a given metabolite, or that convey a signal from the cell surface to the cell nucleus, or that come
Subjects and methods

Subjects and phenotype measurement
Participants in the Irish Affected Sibpair Study of Alcohol Dependence were recruited in Ireland and Northern Ireland between 1998 and 2002. Further details of the study design, sample ascertainment, and clinical characteristics of this sample are described elsewhere. In brief, ascertainment of participants in the Irish Affected Sibpair Study of Alcohol Dependence (AD) and population controls. A total of 130 candidate genes and 186 ancestry informative markers. Our Irish population showed more linkage disequilibrium than the African population for primary alcoholism, which is the most diverse, were obtained from HapMap Phase I Rel18 to re-construct haplotypes for each gene using SNPHAP (http://www.gene.cimr.cam.ac.uk/clayton/software/snphap.txt). A double classification tree search algorithm was applied to select minimum index SNPs representing maximum haplotype information and with frequency > 0.6% for each gene. Probable functional SNPs (non-synonymous, splice site and putative functional SNPs from the literature) were forced in the current Diagnosis and Statistical Manual of Mental Disorders, fourth edition criteria (American Psychiatric Association, 1994) for AD and if all four grandparents had been born in Ireland, Northern Ireland, Scotland, Wales or England. After a prospective family was identified through a proband, parents and potentially affected siblings whom the probands provided permission to contact were recruited.

Probands, siblings and parents were interviewed by clinically trained research interviewers, most of whom had extensive clinical experience with alcoholism. The assessment included demographic characteristics, lifetime history of AD and other comorbid conditions, alcohol-related traits, personality features and clinical records. The Diagnostic and Statistical Manual of Mental Disorders, fourth edition AD diagnosis was assessed in probands and siblings using the modified Semi-Structured Assessment of the Genetics of Alcoholism version 11 (ref. 17) interview to reduce assessment time.

All participants provided informed consent. There were 1238 individuals who completed the Semi-Structured Assessment of the Genetics of Alcoholism interview and met Diagnostic and Statistical Manual of Mental Disorders, fourth edition AD diagnosis, including 591 probands, 620 affected siblings and 27 affected individuals from 10 complex families. Controls were recruited in the Northern Ireland from volunteers donating at the Northern Ireland Blood Transfusion Service (N = 554) and in the Republic from the Garda Siochana (the national police force, N = 38) and the Forsa Cosanta Aituiil (the army reserve, N = 34). Controls were screened and their samples excluded if they reported a history of heavy drinking or problem alcohol use. In the present case–control study design, we included 575 independent AD cases (399 probands and 176 sibs) that were drawn from the Irish Affected Sibpair Study of Alcohol Dependence families and 530 controls, with high yield of high-quality DNA for genotyping.
By construction of the algorithm SNPs in high LD could not both count toward the significance of a gene set.

Genotyping using an Illumina custom SNP array

Genotyping for the addiction array was performed using the Illumina GoldenGate genotyping protocols on 96-well format Sentrix arrays (Illumina, San Diego, CA, USA). All pre-PCR processing was performed using a TECAN liquid handling robot and arrays were imaged using an Illumina Beadstation GX500 (Illumina). The data were analyzed using GenCall v6.2.0.4 and GTS Reports software v5.1.2.0 (Illumina). Full details of array genotyping and post-experiment QC are described elsewhere.\(^1^8\) In brief, a total of 1105 unique samples were genotyped. After screening for poor performing SNPs based on technical metrics from the GX500 and assessment of Hardy–Weinberg equilibrium, the data available for this sample included 1286/1350 (95.3%) of gene SNPs and 176/186 (94.6%) ancestry informative markers. Markers, which passed QC metrics were then assessed for Hardy–Weinberg equilibrium, the data available for this sample included 1286/1350 (95.3%) of gene SNPs and 176/186 (94.6%) ancestry informative markers. Markers, which passed QC metrics were then assessed for Hardy–Weinberg equilibrium, and those with \(P < 0.001\) were discarded. Those SNPs or individuals in which more than 10% values were missing were dropped from further analysis. Any SNPs with minor allele frequency <5% were also discarded.

Identification of signaling gene sets

In this study, we focused on a subset of signaling systems, which were well represented in the available data. We divided up the 130 assayed genes into families based on the signaling systems in which they participate. In most cases, these were sets of receptors for a particular signaling molecule, which annotations were provided with the chip; the genes for corticotropin-releasing hormone (CRH) were selected by DK. This process yielded seven gene sets, which had at least four member genes; several other signaling systems (for example, opioids) had fewer than four genes represented on the chip; these were not considered further for a systems-analysis approach. We concentrated on those seven, which included receptors and other molecules involved in neurotransmission through norepinephrine, acetylcholine, dopamine, CRH, \(\gamma\)-amino butyric acid (GABA), glutamate and serotonin. The genes in each group were as comprehensive as possible limited only by the coverage on the chip. The genes in the gene sets are shown in Table 1.

Statistical approach

Model. Our model is directed toward the Common Disease Common Variant hypothesis. We posit that each variant contributes independently and additively to risk. This can be simply expressed in a logistic regression model: the logit of relative risk is predicted by a weighted sum of counts of risk-bearing alleles as in equation (1):

\[
\log \left( \frac{p}{1 - p} \right) = \sum_{i=1}^{p} \beta_i n_i
\]

where \(n_i\) represents the counts of allele \(i\) and \(\beta_i\) reflects the contribution of allele \(i\) to the phenotype. We are anticipating many small contributions of common alleles to AD risk and therefore anticipate that many of the true coefficients \(\beta_i\) will be non-zero, but not large. Therefore when we fit model (1) to our data, we expect to see that many of the fitted estimates \(\hat{\beta}_i\) of \(\beta_i\) will be significantly different from 0, that is, more fitted coefficients \(\hat{\beta}_i\) will be associated with large increases in deviance explained in model (1), and therefore small \(P\)-values, than we would see in similarly powered studies if there is no underlying effects (if all \(\beta_i\) were 0). To test whether there are more non-zero coefficients than we would expect by chance, we permute the phenotype labels to generate many data sets with the same LD structure among the genotypes, but no consistent relation to the AD phenotype, and ask how unusual is it to find as many significant estimated coefficients \(\hat{\beta}_i\) as in fact occur with the true phenotype labels.

SNP selection. We expect that most typed variants will not be even weakly associated with AD risk, and statistical testing would be weakened by including all variants. We therefore selected subsets of SNPs across gene sets in two ways. First, we followed standard practice for building a multivariate linear model based on increase in log-likelihood. We set a relatively modest threshold of a log likelihood increase of 2.5 for inclusion in a model. Second, we implemented a faster procedure by selecting a subset of alleles that were potentially individually associated with AD using a liberal threshold for inclusion of \(P < 0.20\). Both these inclusion criteria are fairly weak, and statistical significance cannot be assessed by conventional means. For both procedures, we prevented from entering the model those variants in LD \((r^2 > 0.25)\) with variants already in the model.

Statistical significance

To properly assess significance of the AD risk contributions of selected SNPs, therefore, we generated a null distribution by repeating the whole procedure, including SNP selection, 1000 times with randomized phenotype data (for the LR

| Table 1 Genes considered within gene sets |
|------------------------------------------|
| Norepinephrine system                     |
| Cholinergic system                        |
| Dopaminergic system                       |
| GABAergic system                          |
| Glutamatergenic system                    |
| Serotonergic system                       |
| CRH system                                |
| ADRA1A, ADRA2A, ADRA2B, ADRA2C, ADRB2    |
| CHRM1, CHRM2, CHRM3, CHRM4, CHRM5         |
| DRD1, DRD2, DRD3, DRD4, DRD5, DDC, TH     |
| GABRA2, GABRA3, GABRA4, GABRA6, GABRB1,  |
| GABRB2, GABRB3, GABRD, GABRE, GABRG2,    |
| GABRG3, GABRG2, SLC6A7                    |
| GRIK1, GRIN1, GRIN2A, GRIN2B, GRIN2C,    |
| GRM1, HTR1A, HTR1B, HTR2A, HTR2B, HTR2C, |
| HTR3A, HTR3B, CRH, CRHBP, CRHR1, CRHR2   |
inclusion criterion) or 10,000 times (for the faster P-value threshold criterion); for each permutation, we recorded the total deviance. We then compared the total deviance increase for the SNPs chosen using the true outcomes with the distribution of total deviance increases for SNPs chosen using the identical procedure for randomized outcomes; the P-values associated with each gene set was the proportion of permutation fits whose total explained deviance exceeded that of the true fit. We found that the sets of SNPs chosen and the significance of gene sets differ only modestly depending on which criterion for SNP inclusion is used or on which log likelihood threshold is used for the likelihood criterion. We report results for the likelihood criterion, as this is more traditional; the results based on the other inclusion criterion were slightly more significant (not shown).

Results

The individual genes selected for each of the seven gene sets are shown in Table 1 and main results of our analyses are shown in Table 2.

Using the standard Benjamini–Hochberg procedure for FDR, at an expected proportion of 10% of false positives, we identified the following four systems as potential contributors to genetic risk for AD: norepinephrine, glutamate, GABA and CRH. The 27 SNPs in these systems together accounted for 6.8% of the total phenotype variance, although this is likely an overestimate due to selection bias.

If we had done a standard chip-wide association analysis on this data, none of the individual variants in this study would be significantly associated with AD after multiple comparisons corrections. In fact, the smallest Q-value would be 0.7 using the standard Benjamini–Hochberg procedure. However, among our seven gene sets, four gene sets (norepinephrine, glutamate, GABA and CRH) achieved a Q-value <0.1.

To examine the distribution of effect sizes within the positive gene sets, we plotted the odds-ratios (ORs) for all of the SNPs selected by the algorithm in the four systems selected by our procedure (Figure 1). Modest to moderate association signals were distributed widely within both gene sets and were not confined to a few markers. To give some sense of the statistical significance of the odds-ratios associated with these SNPs, for associations with a randomized phenotype, only one in 100 SNPs would have an OR ≥ 1.25. We examined 165 SNPs in the GABA system and 90 in the Glutamate system. Thus, one would expect one or two of the ORs ratios from GABA receptors SNPs to exceed 1.25, whereas we observe nine. For glutamate receptor SNPs, we would expect one and we observe four. For the NE system, we would expect to see 0 or 1 SNP whose OR exceeds 1.25; we see three. For the CRH system, we expect on average to see one SNP whose OR exceeds 1.2 one-half of the time; in fact we see two.

Discussion

The goal of this report was to test a new method for the detection of small effect risk variants for complex neuropsychiatric disorders. We proposed seven gene sets based on a priori biological hypotheses and tested these sets for their impact on risk for AD. Using the standard Benjamini–Hochberg FDR procedure, we identified four gene sets significantly associated with AD with at an FDR of 10%, indicating a low likelihood of their being false discoveries. In contrast, no individual SNP in any of the selected gene sets would have been selected in a chip-wide study at an FDR <40%. If we had hypothesized a priori that our candidate genes would belong to one of the seven groups tested and restricted our single locus tests to those SNPs only, then we would have had only one SNP significant at an FDR of 0.2, (which would also be a Bonferroni-corrected P-value of 0.2), but none at an FDR of 0.15.

Interestingly, the distribution of the association signals in the two largest sets was among several genes rather than being concentrated in one gene. Such a wide distribution of effects would be predicted by the original motivation for this gene set approach.

The validity of our findings is also supported by the work of many previous researchers who have identified the contribution of GABA and glutamate receptors to AD. Some of the genes contained in the gene sets tested here have been previously tested as candidate genes albeit with inconsistent results. Some of the others have yet to reveal association with AD; however, as the effect sizes are small, they would not likely have yielded association when tested individually, even if the association is real. For example,

Table 2  Summary of gene sets and significance of permutation testing

| Genes       | Ach | CRH | Dopamine | GABA | Glutamate | NE | Serotonin |
|-------------|-----|-----|----------|------|-----------|----|-----------|
| Genes       | 5   | 4   | 8        | 13   | 6         | 5  | 7         |
| SNPs assessed| 40  | 22  | 58       | 121  | 65        | 20 | 44        |
| SNPs selected| 2   | 3   | 4        | 13   | 7         | 3  | 3         |
| Nominal P-value | $4.2 \times 10^{-2}$ | $1.1 \times 10^{-2}$ | $2 \times 10^{-3}$ | $1.4 \times 10^{-8}$ | $3.2 \times 10^{-5}$ | $6.7 \times 10^{-3}$ | $1.3 \times 10^{-2}$ |
| Permutation P-value | 0.78 | 0.01 | 0.32     | 0.04 | 0.05      | 0.02 | 0.59      |
| Q-value     | 0.78 | 0.07 | 0.45     | 0.09 | 0.09      | 0.07 | 0.69      |

Abbreviations: GABA, γ-aminobutyric acid; SNP, single nucleotide polymorphism.
previous studies have shown that a number of individual genes for components of the GABA A receptor are associated with AD. Investigations in the Collaborative Studies on the Genetics of Alcoholism sample showed that variations in GABRA2 were highly associated with AD as well as the β-frequency of the electroencephalogram. Another study produced evidence showing gene-environment correlation and interaction in the impact of GABRA2 variants and risk for AD. Other studies have reported association between AD and GABRB3 and severe alcoholism and Song et al found evidence of association with GABRB3. The most frequent combination of GABA A subunits is α1, β1 and δ. Of these three common subunits, only β1 and δ were sampled on the array, but when SNPs for these genes encoding these subunits were considered together as a group they also reached a significance level of 0.025.

There is also increasing evidence that glutamatergic neurotransmission is involved in alcohol tolerance and withdrawal through its role in synaptic plasticity. A limited number of studies testing NMDA receptor subunit genes in human samples have reported association with AD and related phenotypes. Genetic association results for genes coding subunits of the NMDA, kainate and AMPA receptors have produced mixed results despite the substantial functional evidence implicating this system in AD. Results from the current study suggest that other glutamatergic genes also represent putative risk factors for AD.

Although genes in the Norepinephrine and CRH systems have not been tested for AD to the extent that GABA and glutamate have, there is evidence to suggest that the Norepinephrine and CRH systems have a functional role in the etiology of AD. Previous findings have shown that stress system disturbances are observed in AD and are involved in alcohol-induced neurotoxicity and cognitive deficits. The Hypothalamic-pituitary-adrenal (HPA) axis activation influences reward pathways and GABA A receptor function involved in ethanol sensitivity. Evidence for genetic factors is supported by family studies showing altered HPA response to ethanol, and animal studies linking a CRH haplotype in alcohol consumption. Clinical studies show that CRHR1 variants are associated with drinking patterns and CRHBP variants are associated with anxiety-related EEG patterns and depressive symptoms among alcoholics. Findings of HPA axis disturbance in drinking or abstinent AD individuals, however, are inconsistent and difficult to interpret, with both heightened and reduced activity reported. Moreover, acute and chronic alcohol use differ in their effects on HPA axis activity, with disturbed activity in AD potentially due in part to the impact of chronic alcohol consumption on brain regions involved in negative feedback. The CRH system genes may have a role in AD via the effects of the CRH system on the negative affective component in the cycle of addiction or owing to the high comorbidity of AD with major depression (MD).

The fraction of variance accounted for by the subset of SNPs in the GABA receptor genes selected was 3.8%, while the fraction of variance accounted for by the subset of SNPs in the six selected glutamate receptor genes was

![Figure 1](https://example.com/figure1.png)

**Figure 1** Upper left. Effect sizes (as odds-ratios) for SNPs selected in the GABA gene set. Three SNPs for GABA occur in the same gene (GABRG3), but the markers are not in LD ($r^2 < 0.25$ by construction). Upper right. Odds ratios for SNPs in the glutamate receptor gene set. Lower left. Odds ratios for selected SNPs in the Norepinephrine gene set. Lower right. Odds ratios for SNPs selected in the CRH gene set. For the GABA system at upper right the prefixes SLC (for ‘6A7’ only) and GABR (for the rest) have been omitted from gene names in the bottom row.
3.3%. We note that these estimates are comparable with previously published estimates for the contributions of multiple variants in distinct neurotransmitter systems to a different psychiatric phenotype (attention deficit hyperactivity disorder). For comparison purposes, the fraction of variance that would be explained by comparably sized subsets of SNPs, randomly selected from those of comparable minor allele frequencies, would be 1.1 and 0.6%, respectively. We think that a systematic search of other gene sets (including those related to metabolism of alcohol and taste) along the lines performed here may yield further useful results.

One concern about this study may be that the genes were a priori selected to be likely candidates for association with addictive behavior. We agree that this means it is more likely that we have higher levels of association than in a GWAS. However, this caveat applies equally well to the individual tests, which as noted above, do not result in experiment-wide significance, and in any event a priori selection does not invalidate the statistical evidence of an independent study.

This method is likely to prove most useful in complex biomedicological phenotypes where information exists on plausible pathophysiological mechanisms. Particularly when applied to genome wide data, the task will be to develop informed sets of candidate pathways that can be tested as plausible a priori hypotheses. Defining the boundaries of individual pathways may prove challenging as biological pathways are rarely discrete. Nonetheless, if the common disease/common variant hypothesis is correct, genetic variation for many common diseases may be scattered throughout a number of genes within particular biological pathways. Testing a limited number of such pre-defined pathways is likely to lead to a substantial gain in statistical power if it is possible to nominate the likely candidate pathways. Such a gain in power could significantly reduce the need for very large samples for GWAS needed to compensate for the multiple testing burden.

In summary, we see the most important results here as methodological. We have developed and tested a network-based approach to clarifying the genetic substrate of complex diseases. Applying this to a pre-selected set of candidate genes genotypes in cases with AD and controls, we found evidence that variants in four neurotransmitter pathways (norepinephrine, glutamate, GABA and CRH) significantly contributed to risk for AD. These results are broadly consistent with the previous literature and suggest that network analyses may be a useful addition to the more standard approaches to clarifying the genetic basis of complex disorders, especially when previous biological hypothesis of disease etiology can lead to predicted gene pathways.

Conflict of interest

The authors declare no conflict of interest.

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