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Possible involvement of the circadian pathway in alcohol use disorder in a South African adolescent cohort

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Abstract

Alcoholism has an estimated heritability of between 40–60% and it is thought that several genes of small effect may contribute to the risk of developing this disorder. Studies of the genetics of alcohol use disorder (AUD) may, however, be confounded by issues of comorbidity. The aim of this investigation was to assess associations between variants in a range of candidate genes and AUD in a unique sample of adolescents without comorbidity. Our cohort consisted of 80 adolescents with an AUD diagnosis and 80 matched controls of mixed ancestry ethnicity. An Illumina Infinium iSelect custom 6000 bead chip was used to genotype 5348 SNPs in 378 candidate genes. Association analysis, gene-based analysis and polygenic scoring were performed. There was no statistical association between any of the investigated SNPs and AUD after correction for multiple testing. However, from the gene-based analysis it was found that the circadian rhythm genes NR1D1 and BHLHE41 are associated with AUD. While preliminary, these data provide some evidence that the circadian pathway may be relevant to the pathophysiology of AUDs. Study of early onset non-comorbid populations with AUD may be useful in identifying target genes for study in larger more representative samples.

Keywords

Alcoholism; Single Nucleotide Polymorphism; Circadian

Introduction

Twin studies have shown that alcohol use has a considerable genetic influence (Prescott and Kendler 1999) with an estimated heritability of between 40–60% (Epps and Holt 2011). A recent study has shown that individuals with one parent affected with an alcohol use disorder (AUD) have a 2.5 fold increased lifetime odds of developing an AUD themselves. In

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addition, those individuals with two affected parents have a 4.4 fold increase in the odds of developing a lifetime AUD and females are more vulnerable than males to the effect of a parent with an AUD (Yoon et al. 2013). Many genes (approximately 670) and variants have been implicated in alcoholism (http://www.hugenavigator.net/HuGENavigator/phenoPedia).

Comorbidity is highly prevalent in psychiatric disorders. A previous study found that 37% of individuals with an AUD had mental disorder comorbidity (Regier et al. 1990). Several studies have investigated the genetic basis of AUD comorbid with other psychiatric disorders (Hu et al. 2013, Lydall et al. 2011, Prescott et al. 2000, Yasseen et al. 2009). In addition, genetic variants relevant to other psychiatric disorders have also been associated with AUD (Schumann et al. 2011). These studies suggest that there exists considerable overlap in the genetic aetiologies of various psychiatric disorders. Therefore, it is of interest to identify and investigate genetic polymorphisms which may potentially be common between AUD and other psychiatric disorders. However, comorbidity may well confound studies of genetic aetiology (Merikangas and Swendsen 1997). In this regard it is particularly important to investigate the genetic aetiology of AUD in individuals with AUD without psychiatric comorbidity.

To attain a more genetically homogenous group and minimise the confounding effect of comorbidity, a group of adolescents with AUD but without any psychiatric comorbidity or poly-substance use were investigated. The aim of this investigation was to determine whether SNPs previously found to be associated with other psychiatric disorders are associated with AUD in an adolescent South African cohort. This was achieved by genotyping adolescents affected with AUD and matched controls using a custom Illumina Infinium iSelect custom 6000 bead chip and analysing with both single-SNP and gene-based association tests.

Methods

Participants

Ethical approval for this study (including the consent form used in the recruitment procedure) was obtained from the Research Ethics Committees of Stellenbosch University (N06/07/128) and the University of Cape Town (HREC REF 023/2012). Written informed assent was obtained from each of the study participants and written informed consent was obtained from their parents or guardians. The consent form contained full details of all study related activities in the language understandable by the study population. The research group consisted of 80 adolescents with DSM-IV AUD, and 80 age- and gender-matched light (lifetime dosage not exceeding 80 units of alcohol)/non-drinking controls (HC). Eligibility was assessed after a detailed medical history was taken, physical and psychiatric examinations. In addition, participants underwent urine analysis and breathalyser testing to ensure they were not intoxicated during the testing period. To determine whether any of the participants had current or past psychiatric symptoms, the Schedule for Affective Disorders and Schizophrenia for School Aged Children (6–18 years) Lifetime Version (K-SADS-PL) (Kaufman et al. 1997), a semi-structured, clinician administered, diagnostic interview was used. In addition to the K-SADS-PL, the Timeline Followback (TLFB) procedure, a semi-

structured, clinician administered assessment, was used to determine lifetime history of alcohol use and drinking patterns (Sobell and Sobell 1992).

Exclusion criteria for study participation included diagnoses of mental retardation, lifetime DSM-IV Axis I other than AUD; lifetime dosages exceeding 30 cannabis joints or 4 methamphetamine doses; current use of sedative or psychotropic medication; signs or history of fetal alcohol syndrome or malnutrition; sensory impairment; history of traumatic brain injury with loss of consciousness exceeding 10 minutes; presence of diseases that may affect the CNS; less than 6 years of formal education; and lack of proficiency in English or Afrikaans. Blood samples were collected for all of these individuals with the appropriate written informed consent.

Genotyping

An Illumina Infinium iSelect custom 6000 bead chip was used to genotype 5348 SNPs in 378 candidate genes (genes involved in neurotransmitter and neuroendocrine systems) for post-traumatic stress disorder, and SNPs and copy number variation (CNVs) which were "significant hits" from previous psychiatric GWAS studies. The bead chip was run on the Illumina BeadStation 500G System at the University of Michigan DNA Sequencing Core (Michigan, USA). Case and control samples were analysed together. Genotype calls were made using standard clustering algorithms in the GenomeStudio software (Illumina).

Statistical Analysis

Identity by State (IBS) Clustering—For case-control association studies, false positive results may be observed due to the presence of population stratification. Population stratification is when there are differences in the frequencies of ethnic groups in cases and controls and thus a difference in allele frequencies not due to the disease/phenotype of interest. This may also occur in admixed population groups, whereby "different fractions of ancestry from each ancestral sub-population are observed". Historically this has been overcome by the careful matching of cases and controls and the use of family-based association studies (Enoch et al. 2006).

In this study, cases and controls were matched but in addition to this, IBS clustering was performed using the whole genome association analysis toolset, Plink (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell et al. 2007), in order to account for possible population stratification within the mixed ancestry group. Using pairwise identity by state (IBS) distance, this process uses a linkage agglomerative clustering method to determine whether two individuals belong to the same or different population groups. This test was run without any constraints.

Association Testing: Single SNP—Using Plink, logistic regression was used to test whether any of the SNPs had an association with AUD. All samples had a call rate of greater than 99%. Before genotyping and frequency pruning there were 4656 SNPs. A total of 9 SNPs failed the missingness test (i.e. only SNPs with a genotyping rate of 90% were included) and 583 SNPs were excluded because of a minor allele frequency (MAF) of less than 0.05. A total of 4 SNPs were excluded as they were found to be out of Hardy-Weinberg

Equilibrium (HWE) (p-value less than 0.00001). IBS cluster ID was included as a covariate and all tests were adjusted for multiple testing using the Bonferroni test.

Association Testing: Gene-based—A gene-based test was performed in Plink whereby SNPs from the same gene were grouped together and tested for association to AUD. Intergenic and control SNPs were excluded from the analysis. Gene-based tests are calculated in the following way: 1) determine which SNPs are in LD above a specified R threshold; 2) single SNP association to phenotype is carried out; 3) "independent" SNPs are selected (as defined by step 1) which are lower than the specified p-value. SNPs are selected in order of decreasing statistical significance, after removing SNPs in LD with previously selected SNPs; 4) a set statistic is calculated as the mean of the single SNP statistics; 5) the dataset is permuted at the specified number of times; 6) steps 2 to 4 are repeated for each permutation; 7) the *EMP1* p-value is the number of times the permuted set statistic is higher than the original p-value calculated for that set.

A logistic regression test was run with cluster assignment as a covariate. SNPs with a MAF of less than 0.05 and a missingness rate of greater than 90% were excluded. A total of 10 000 permutations were run, with the default values for r^2 (0.5), p-value (0.05) and the maximum number of SNPs (5).

Polygenic Profile Scoring—The polygenic scoring procedure was performed to determine whether there was overlap in common "risk" variants of small effect, between AUD and other psychiatric disorders. This method was first proposed by Purcell et al. (2009) and the basis of the procedure followed described below (Purcell et al. 2009). Firstly, data was obtained from a "discovery" dataset. In this instance, genotyping data for bipolar disorder (BD), major depressive disorder (MDD) and schizophrenia (SCZ) were obtained from the Psychiatric Genetics Consortium (PGC) website. (https://pgc.unc.edu/Sharing.php#SharingOpp) (Date accessed: 19 October 2012) Secondly, SNPs were selected which overlapped between the Illumina bead chip used in this study and the three PGC datasets; and were below a certain p-value significance threshold (P_T). The number of overlapping SNPs in each dataset, at varying thresholds, are listed in Table 1. The following formula was used to calculate the polygenic profile score:

$$\text{Polygenic Profile Score} = S_1 * W_1 + S_2 * W_2 + S_3 * W_3 + \ldots n_i$$

(S_i= no. of reference alleles the individual has at SNP_i, W_i= log odds ratio of SNP_i)

This score was calculated using the "score" option in Plink. Logistic regression was conducted to test whether the generated polygenic score for each individual had an association with AUD. This was done using the statistical environment R (http://www.r-project.org/).

Results

Participants

The median ages of the AUD and HC groups were 14.94 (IQR 15.53–14.47) and 14.92 (IQR 15.33–14.36), respectively. In total, there were 47 females and 33 males in both the AUD and HC groups. The median number of lifetime alcohol dose units for the AUD and HC groups were 1125.50 and 1.0, respectively, where a unit refers to one beer or wine cooler, one glass of wine, or one 43g shot of liquor (on its own, or in a mixed drink). The majority of the study participants were Afrikaans speaking and the median number of years of education was 8.0 years for both groups (AUD and HC).

Statistical Analysis

IBS Clustering—The cohort was divided into 4 clusters, indicating population stratification within our mixed ancestry group. The cluster assignment for each individual was used as a covariate in subsequent association testing. The biggest and smallest clusters consisted of 65 and 14 individuals, respectively.

Association Testing: Single SNP—Logistic regression was used to test whether any of the SNPs had an association with AUD (cases vs. HC). A total of 192 SNPs obtained a p-value of less than 0.05. The SNP with the smallest p-value was rs7105258 (p-value=0.0018), located in the gene glutamate receptor, ionotropic kainate 4 (GRIK4) (chr11q22.3). The SNPs with the second- and third-most significant p-values were rs2071570 (p-value=0.002) located in the gene nuclear receptor subfamily 1, group D (NR1D1) (chr17q11.2) and rs11030119 (p-value=0.002) located in the gene brain-derived neurotrophic factor (BDNF) (chr11p13), respectively. After correction for multiple testing, none of these SNPs remained statistically significant.

Association Testing: Gene-based—The gene which obtained the most significant association to AUD was *NR1D1* (p-value=0.003), consisting of 5 SNPs in the set, with 1 SNP being significant (rs2071570). The second-most significant SNP (p-value=0.006) is located in the gene basic helix-loop-helix family, member e41 (BHLHE41, also known as BHLHB3 and DEC2). This set consisted of four SNPs, with rs3809140 being the only significant SNP in the set. Both of the above-mentioned genes form part of the circadian pathway.

Polygenic Profile Scoring—From the logistic regression analysis, none of the polygenic scores generated for each of the individuals in the cohort had an association with AUD. Table 1 lists the p-values obtained for each disorder at each p-value threshold.

Discussion

In this study there were no statistical associations between any of the investigated SNPs and AUD after correction for multiple testing. This finding is not unexpected given that multiple genes of small effect may influence AUD, and given the relatively small sample size. From the gene-based analysis, however, the circadian rhythm genes NR1D1 and BHLHE41 were associated with AUD.

Both NRID1 (also known as REV-ERBα) and BHLHE41 play essential roles in the circadian pathway (the protein encoded by the BHLHE41 gene is a transcriptional repressor) (Fujimoto et al. 2007). This is consistent with animal and human evidence that circadian disturbances may be relevant to AUD (Chen et al. 2004, Hatonen et al. 2008). NR1D1 is an orphan nuclear hormone receptor gene which is a “gatekeeper” for appropriate co-ordination of the circadian response (Duez and Staels 2009) by regulating the transcription of the clock gene BMAL1 (Preitner et al. 2002). This is the first time this particular gene variant has been associated with AUD but it has been associated with BD (Kripke et al. 2009) and it may well play a role in a range of neuropsychiatric disorders given the importance of circadian rhythms in a variety of psychobiological processes (Merrow et al. 2005). Similarly, BHLHE41 which is rhythmically expressed in the suprachiasmatic nucleus, is involved in the repression of the CLOCK/BMAL1 heterodimer dependent transcription of the PER1 gene (Honma et al. 2002). As with NR1D1, this gene and variant has also not previously been implicated in AUD.

Athough no single SNP association reached statistical significance, it is worth commenting that the most significant SNP was the rs7105258 intronic SNP, located in the glutamate ionotropic receptor gene, GRIK4. While this is the first time that this particular SNP and gene have been associated with AUD, previous studies have highlighted the role of the glutamate system in AUDs, with variation in the glutamate receptor, ionotropic, kainite 1 (GRIK1) gene associated with alcohol dependence (Kranzler et al. 2009) and variants in the glutamate receptor, ionotropic, N-methyl D-aspartate 2A (NR2A) gene have been shown to have an association with alcohol dependence, positive family history, an earlier age of onset, risky drinking patterns in adolescents and maximum number of drinks for adults (Schumann et al. 2008). Further work with larger samples is needed to explore the role of GRIK4 in particular.

Indeed, an important limitation of this investigation is the small sample size. For this study to have a power of 90%, an approximately seven times larger sample size would be required. Nevertheless, even a small sample may be useful in providing signals for further investigation. The value of this sample lies in the early onset of the phenotype, and in the lack of comorbidity. However, this does not preclude these adolescents from developing other psychiatric diagnoses and the controls from developing AUD, in the future. Also, while the identified genes NR1D1 and BHLHE41 are certainly involved in the circadian pathway, these genes also form part of other cellular-molecular functions such as adipogenesis (Chawla and Lazar 1993) and regulation of apoptosis (Liu et al. 2010). Thus there is no definitive evidence that the association of these genes to AUD implicates circadian clock disruption in this disorder.

If the findings here are replicated, then it may well be useful to investigate the circadian and possibly glutamatergic pathways in greater detail by performing gene expression and epigenetic studies for each respective pathway in larger AUD cohorts. Also, the relationship between the circadian and glutamate pathways in alcoholism should be further investigated given some prior evidence that this is relevant (Spanagel et al. 2005). In conclusion, while preliminary and tentative, these data are from a unique population, and provide some evidence that the circadian pathway may be important when considering the aetiology of

AUDs. Understanding the biology of AUD may ultimately aid in the identification of “at risk” individuals and enable the tailoring of personalised treatment.

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Table 1

Polygenic profile scoring results for AUD and 3 PGC datasets

p-value significance threshold	Bipolar Disorder		Major Depressive Disorder		Schizophrenia	
	No. SNPs	p-value	No. SNPs	p-value	No. SNPs	p-value
All	3528		2470		2479	
< 0.1	576	0.71	291	0.53	424	0.93
< 0.2	940	0.76	557	0.45	693	0.41
< 0.3	1319	0.89	796	0.41	941	0.59
< 0.4	1670	0.79	1017	0.97	1156	0.72
< 0.5	1993	0.83	1259	0.98	1374	0.62