Interaction of Multiple Gene Variants and their Effects on Schizophrenia Phenotypes

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Highlights

- Schizophrenia is a clinically heterogeneous disorder attributed to complex genetics
- Inconsistent genetic association studies may be due to phenotype differences
- 50 SNPs from 21 schizophrenia genes were selected for phenotype association study
- 3 clusters were found characterised by speech, hallucination and delusion symptoms
- Different SNP sets were associated with different schizophrenia phenotype clusters

Abstract

Background: Schizophrenia is a clinically heterogeneous disorder and may be explained by its complex genetic architecture. Many schizophrenia susceptibility genes were identified but the picture remains unclear due to inconsistent or contradictory genetic association studies. This confusion may, in part, be because symptoms result from the combined interaction of many genes and these interacting genes are associated with specific sub-phenotypes of schizophrenia rather than schizophrenia as a whole. This study investigates the relationship between schizophrenia susceptibility genes and schizophrenia sub-phenotypes by identifying multiple gene variant interactions.

Materials and Methods: 50 SNPs from 21 genes were genotyped in 235 Australian participants with schizophrenia screened for various phenotypes. Schizophrenia participants were grouped into relevant phenotype clusters using cluster analysis and normalised phenotype cluster scores were calculated for each patient. The relationship between genotypes and normalised phenotype cluster scores were analysed by linear regression analysis.

Results: Three phenotype clusters were identified. There was some overlap in symptoms between phenotype clusters, particularly for depression. However, cluster 1 appears to be characterised by speech disorder and affective behaviour symptoms, cluster 2 has predominantly hallucination symptoms and cluster 3 has mainly delusion symptoms. Interaction of five SNPs was found to have an effect on cluster 1 symptoms; ten SNPs on cluster 2 symptoms; and eight SNPs on cluster 3 symptoms.

Conclusion: The interaction of specific susceptibility genes is likely to lead to specific clinical sub-phenotypes of schizophrenia. Larger patient cohorts with more extensive clinical

data will improve the detection of gene interactions and the resultant schizophrenia clinical phenotypes.

1 Introduction

Schizophrenia is a heterogeneous disorder consisting of multiple symptoms that include delusions, hallucinations, thought disorders, speech disorders, diminished emotion and neuromuscular dysfunction. The disorder is known to be caused by dysfunction in neurotransmitter systems, particularly the dopamine and glutamate systems [1]. Genetics is known to be a large component that contributes to schizophrenia development as the heritability estimates range from 65-80% [2]. Studies have identified many genes that are associated with schizophrenia that also have roles in neurotransmission [3-7].

Numerous association studies have identified a large number of schizophrenia susceptibility genes using both candidate gene and genome-wide association study approaches. Genetic association studies are inconsistent, with many reports failing to validate results for genes. This is even when the genes are quite well established as schizophrenia susceptibility genes, e.g. *BDNF* [8, 9] and *NOS1AP* [10, 11], or as neurotransmission related genes, including *DTNBP1* [12, 13]. Schizophrenia is a clinically heterogeneous disorder with patients presenting with a wide variation in both the nature and severity of symptoms. It is thought that this heterogeneity is due to the underlying genetic complexity involving numerous genes. It is possible that clinical differences between sub-groups of those with schizophrenia may be responsible for the inconsistencies seen in schizophrenia association studies. This raises the question of whether some schizophrenia susceptibility genes may be associated with specific phenotypes of schizophrenia rather than schizophrenia as a whole.

Several psychosis related phenotypes have been found to be associated with variants of schizophrenia susceptibility genes. One of the phenotypes includes poor working memory which was found associated with numerous genes including *COMT* [14], *RGS4* [15], *COMT*-

GRM3 interaction [16], *ZNF804A* [17] and *CACNA1C* [18]. *NOS1* was found to be associated with cognitive control and attention [19]. *DRD2* [20], *COMT* [21] and *MIR137* [22] were also found to be associated with emotion processing. Additionally, our previous studies found that *BDNF* [23], *DTNBP1* [24], and *NOS1AP* [25] were associated with clinical phenotypes within schizophrenia; including alcohol dependence, hallucinations and depression. These findings add weight to the concept that, not only is schizophrenia itself a complex genetic disorder but much of the clinical heterogeneity may be due to the involvement of different and possibly unique sets of susceptibility genes in particular groups.

The current study aims to investigate the role of multiple gene variants and their combined effects on specific phenotypes of schizophrenia.

2 Materials and Methods

2.1.1 Schizophrenia Subjects

The subjects were obtained from the Australian Schizophrenia Research Bank (ASRB: http://www.schizophreniaresearch.org.au). The ASRB facilitates schizophrenia research by collecting, storing and providing comprehensive clinical and genetic data from people with schizophrenia and healthy controls. Subjects consisted of 235 schizophrenia patients of European descent with a mean age of 43.9 years (s.d. ± 10.7 years). There were 70 females and 165 males with a confirmed diagnosis of schizophrenia according to DSM-IV/ICD-10 diagnostic criteria with a mean onset age of 23.18 years (s.d. ± 6.31 years). All participants underwent a clinical and neuropsychological assessment and provided a blood sample at the time of assessment. The participants were assessed using a clinical assessment battery that consists of the Diagnostic Interview for Psychosis (DIP) [26] to collect clinical measures including depression, mania, hallucinations, subjective thought disorder, delusions, behaviour and affect, and speech disorder. The patients were recruited from several sources across five Australian States and Territories (New South Wales, Australian Capital Territory, Queensland, Western Australia and Victoria) using media advertisements, inpatient, outpatient and community mental health service providers, non-government organisations and rehabilitation services.

2.1.2 Control Subjects

The control group was also obtained from the ASRB. The controls consisted of 121 females and 104 males of European descent, with a mean age of 45.00 years (s.d. ± 13.2 years). Healthy controls were screened for a family history of, or treatment for, a psychiatric illness

at the time of registration. The controls were assessed using a clinical assessment battery that consists of the Diagnostic Interview for Psychosis (DIP) [26] to collect socio-demographic, family and medical history data.

2.2 Ethics

Each participant gave written informed consent before commencement of data collection. Ethics approval for the project was obtained from the Human Research Ethics Committee of the Queensland University of Technology. This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3 Genotyping

DNA was provided by the ASRB. Twenty-one candidate genes were selected because of either their previous association with schizophrenia, or being part of the dopaminergic or glutamatergic pathways (Table 1). A total of 51 SNPs (single nucleotide polymorphisms) were genotyped across the 21 genes. SNPs were chosen based on their location in functional domains or because they were tag SNPs in haplotype blocks. Tag SNPs were selected using HapMap project phase 2 with a minor allele frequency cut-off of 0.15 and were identified using the pair-wise option of Tagger with $r^2 > 0.8$ threshold.

Samples were genotyped using a homogeneous MassEXTEND (hME) Sequenom assay performed by the Australian Genome Research Facility as previously described [27].

2.4 Data

Data used for analysis include clinical data and genotype data. Clinical data consist of 44 binary measures (Supplementary Table 1) marking the presence (score 1) or absence (score 0) of a defined psychological phenotype. Genotype data consist of imputed data of three genotypes per SNP for 50 SNPs (after removing one SNP, rs528528 from the *RELN* gene, which could not be imputed due to high missing values (>10%) and was excluded from analysis). Missing genotypes were imputed using the Markov Chain Monte Carlo method for 1000 iterations.

2.5 Analysis

2.5.1 Schizophrenia-SNP Association Test

Power calculations for SNP-schizophrenia association was performed using Mann-Whitney power calculator from the Compare2 software package [28] that screens power from genotype frequencies in a pool of schizophrenia and controls at a desired odds ratio. Each of the 50 SNPs was tested for association with schizophrenia by comparing genotype frequencies between schizophrenia patients and controls using the likelihood-ratio chi-square test. Correction for multiple testing of SNP-schizophrenia association was performed using the Benjamini-Hochberg method [29]. Additionally, the SNPs were also tested for their interaction effects on schizophrenia using binary logistic regression.

2.5.2 Phenotype Clustering

Forty four phenotype measures were clustered with Ward's hierarchical clustering method using Statistical Package for the Social Sciences (SPSS) 21. The number of clusters was justified based on dendrogram and coefficient change in the agglomeration schedule.

2.5.3 Assigning Phenotype Cluster Membership to Patients

Based on the number of clusters obtained from phenotype clustering, each of the 235 schizophrenia patients were assigned with membership of a cluster using Ward's hierarchical clustering method.

2.5.4 Identifying SNP-SNP Interaction Associated with a Phenotype Cluster

Association between SNP-SNP interaction and phenotype clusters was identified using linear regression. Genotypes from 50 SNPs were regressed against cluster scores for all resulting clusters using backward elimination linear regression. Resulting models from regressions were subsequently compared to identify the best SNP-SNP interaction model that could explain their respective cluster.

3 Results

3.1 Schizophrenia SNP Association

Power calculations for SNP-schizophrenia association revealed that SNPs with minor genotype frequency of 5% have 80% chance to detect an association with schizophrenia at 1.67 odds ratio (rs17470454, rs1800497, rs1800499, rs2273816, rs2711844, rs324035, rs362691, rs4263535, rs450046, rs5747933, rs6265, rs6314, rs6675281 and rs7103411 have <5% minor genotype frequency). Only 3 out of 50 SNPs were associated with schizophrenia but were not significant after correction for multiple testing, i.e. *NOS1AP* rs1858232 (P = 0.035, q = 0.001), *DDC* rs1966839 (P = 0.046, q = 0.003) and *DRD3* rs324035 (P = 0.038, q = 0.002). However, SNP interaction analysis with backward elimination binary logistic regression detected a 10-SNP interaction model with an overall logistic regression P value of 0.004539 (Table 2). The higher sensitivity to detect association seen when using the interaction of combined SNPs led to the analysis of the combined effect of SNP on more defined sub-phenotypes of schizophrenia.

3.2 Phenotype Clustering

Forty four phenotypes were subjected to a clustering procedure and a dendrogram was generated (Figure 1) using Ward's method. The agglomeration schedule showed the largest coefficient change for two clusters (Ward's linkage coefficient change = 278.088) compared to three clusters (Ward's linkage coefficient change = 88.764). The two-cluster and three cluster solutions can be seen in the dendrogram (Figure 1) where the very large upper cluster

is subdivided into two to provide a total of three clusters. The three-cluster solution was chosen for further analysis as it provides greater discrimination between schizophrenia sub-phenotypes than two clusters.

Each schizophrenia patient was subsequently assigned membership to their respective cluster. While the phenotype clusters contained some common symptoms like depression, there were some characteristic features. Cluster 1 consists of 16 phenotypes (top of dendrogram in Figure 1) and is characterised by speech disorder and affective behaviour symptoms. Cluster 2 consists of 20 phenotypes (middle of dendrogram in Figure 1) with predominantly hallucinations as its characteristic feature. Cluster 3 consists of 8 phenotypes (bottom of dendrogram in Figure 1) and is best characterised by delusion symptoms. There was higher proportion of males compared to females in all three clusters (cluster 1, 62% males; cluster 2, 63% males; cluster 3, 71% males). Analysis accounting for gender effects was not performed due to sample size constraints and the categorical nature of gender variable.

3.3 Linear Regression: Identifying SNP-SNP Interaction within Phenotype Clusters

Backward elimination linear regression was performed between 50 SNP genotypes and normalised scores from three phenotype clusters. Normalised cluster scores were calculated for each patient based on the sum scores of phenotypes within each cluster for all three clusters. For regression between 50 SNPs and normalised cluster 1 scores, backward elimination linear regression concluded with a 5-SNP interaction model with an overall regression *P*-value of 0.000001 (Table 3). This suggests that SNPs rs2711844, rs410557,

rs4656355, rs1966839 and rs1049353 (in order of largest to smallest effect size) are likely to be interacting elements that result in cluster 1 phenotypes.

For regression between 50 SNPs and normalised cluster 2 scores, backward elimination linear regression resulted in a 10-SNP interaction model with an overall regression *P*-value of 0.001303 (Table 4). This suggests that SNPs rs1468412, rs450046, rs2273816, rs2282956, rs1415259, rs6675281, rs4531275, rs1049353, rs4656355 and rs2711844 are very likely to be interacting elements that contribute to cluster 2 phenotypes.

For regressions between 50 SNPs and normalised cluster 3 scores, backward elimination linear regression resulted in an 8-SNP interaction model with an overall regression *P*-value of 0.003342 (Table 5). This suggests that SNPs rs2734839, rs386231, rs1858232, rs2282965, rs6314, rs6347, rs4680 and rs1966839 are very likely to be interacting elements that cause the phenotype pattern seen in cluster 3.

4 Discussion

Our analysis has identified three clusters of phenotypes within our Australian schizophrenia cohort. Depression seems to be a common feature of all three clusters but each cluster can be described by characteristic features, i.e. affective behaviour and speech related symptoms, hallucinations and delusions. After regression of 50 SNPs against the three phenotype clusters it was clear that several genes were important for symptom development. Four out of five genes in symptom cluster 1 are also common to cluster 2 (*NOS1AP*, *CNR1*, *PRODH* and *RELN)*. This may be due to the common phenotypes (depression, subjective thought disorder, behaviour and affect disorder, and delusions) that are shared in both clusters. An interesting observation was that one gene found in all three clusters was *NOS1AP*which has previously been linked to depression [25, 30]. It is not surprising to find behavioural and speech related symptoms in the same symptom cluster (cluster 1), as behavioural problems are known to be risk factors of speech related symptoms [31, 32].

Single SNP association analysis was only able to detect 3 SNPs that were marginally associated with schizophrenia but they did not survive multiple testing correction. However, after employing interaction analysis, a combination of 10 SNPs were highly associated with schizophrenia ($P = 0.004539$). This validates the approach of analysing the interaction of several SNPs in schizophrenia phenotypes and indicates that it is a more powerful approach for detecting SNPs with small effect sizes which would not normally be detected by standard association analysis. One interesting observation is that of the three SNPs found associated with schizophrenia, only two were detected in the 10-SNP interaction effect in schizophrenia but not rs324035 *(DRD3*). Though there is a possibility that the effect of rs324035 on schizophrenia is independent of the 10-SNP set interaction, a more plausible explanation is

that association of rs324035 may be a coincidental finding since its association is not significant after correction for multiple testing.

The *COMT* variant rs4680 which is associated with cluster 3 was previously reported to be associated with depression related phenotypes including major depressive disorder (MDD) [33], decreased positive affect in depression [34], decreased brain volume in MDD patients [35], and *COMT-MTHFR* interaction in MDD patients [36]. One study identified the association of rs4680 with delusions and aggression [37]. Other genes that we found associated with cluster 3 symptoms including *NOS1AP* [30], *DAT* [38, 39], *DDC* [40], and *GRM3* [41, 42] were found to contribute towards depression and delusion related phenotypes in other studies. This further supports the involvement of *COMT*, *NOS1AP*, *DAT*, *DDC* and *GRM3* in a joint interaction that are associated with depression and delusion related phenotypes of schizophrenia.

The genes identified to be cumulatively associated with cluster 1 symptoms (speech) are in agreement with a number of studies which identified the involvement of *RELN* [43]*, CNR1* [44] and *PRODH* [45] in speech related disorders although the involvement of *NOS1AP* and *DDC* has yet to be identified by other studies to further support our findings. There is no clear explanation for the mechanisms of *RELN, CNR1* and *PRODH* in speech. However, the functional deficits related to *RELN* (neuroanatomical and social interaction deficits [46, 47]), along with *CNR1* (involved with neurotransmission and expressed abundantly in brain regions implicated with speech function [48, 49]) and *PRODH* (general neurotransmission [50, 51]) suggest an overall neurological dysfunction that potentially results in speech related symptoms. As for the interacting gene set in cluster 2 symptoms (hallucinations), genes identified to associate directly with hallucinations include *PRODH* [52] and *DISC1* [53, 54];

while the role of *CNR1*, *GRM3*, *KPNA3*, *RELN* and *NOS1AP* in hallucinations remains unclear. Perhaps the dysfunction of *PRODH* and *DISC1* are likely to initiate the pathophysiology leading to hallucinations in schizophrenia, while the remaining genes in cluster 2 are likely to serve as "catalyst" and further exacerbate the hallucinations pathophysiology via abnormal neurotransmission and neurodevelopment activities. Regarding cluster 3 (delusions), genes identified to have direct association with delusions include *HTR2A* [55, 56], *SLC6A3* [38], *COMT* [37, 57, 58] and *DRD2* [59] which are all commonly involved in dopaminergic neurotransmission, its regulation and cognition (related to delusions). Though the role of *GRM3*, *DDC* and *NOS1AP* in delusions is unclear, it is likely that they act as intermediates rather than initiators of the pathology leading to delusions; or associated with other general schizophrenia symptoms.

The study by Arnedo et al. like ours also identified gene sets associated with distinct clinical symptoms which demonstrates that schizophrenia is a heterogeneous disorder [60]. Although the Arnedo et al. study was a GWAS study with larger sample size and SNP numbers they performed a similar analysis which also involved clustering symptoms based on symptom similarities among cases and associating symptom clusters with SNPs [60]. They demonstrated that combining genotypic data with comprehensive phenotypic data greatly increases the power of a study.

5 Conclusion

Overall, results suggest the potential involvement of combined effects from *NOS1AP, CNR1, PRODH, RELN* and *DDC* variants with the pathology of speech disorder in schizophrenia; while the combined effects of *NOS1AP, CNR1, PRODH, RELN, GRM3, KPNA3* and *DISC1* variants are potentially involved with the pathology of hallucination symptoms in

schizophrenia. The combined effects of *NOS1AP, DDC, GMR3, DRD2, COMT, HTR2A* and *DAT* variants are potentially involved with the pathology of depression and delusion symptoms. A larger sample size of schizophrenia patients with uniformly and objectively defined phenotypes may achieve clearer and more characteristic clustering results, which would allow SNPs to be more accurately correlated with their relevant sub-phenotypes and biology. Results should also be interpreted with caution as gender effects have not been included in the multi-SNP interaction models.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

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List of Tables

Table 1: Genotyped SNPs.

Gene	SNPs				
CNR1	rs1049353				
DAT	rs11133767	rs40184	rs4975646	rs6347	
NOS1AP	rs1415259	rs1415263	rs1858232	rs386231	rs4531275
	rs4656355	rs4657178	rs6683968	rs6704393	
GRM3	rs1468412	rs2282965			
COMT	rs165774	rs4646316	rs4680		
DTNBP1	rs17470454	rs1997679	rs4236167	rs9370822	rs9370823
GABRA1	rs17545383	rs4263535			
ANKK1	rs1800497				
DRD2	rs1800499	rs2734839	rs6277		
DRD3	rs1800828	rs324035			
DDC	rs1966839	rs2329371			
PRODH	rs2238733	rs410557	rs450046	rs5747933	
KPNA3	rs2273816				
RELN	rs2528856	rs2711844	rs362691	rs528528	
NRG1	rs3924999				
BDNF	rs6265	rs7103411			
HTR2A	rs6314				
DISC1	rs6675281				
GRIN2C	rs690533				
G72	rs7139958				
GRIN2B	rs7301328				

Table 2: 10-SNP interaction model and their contribution towards schizophrenia.

SNP	Gene	Effect size[1]	*P*-value
rs2329371	*DDC*	0.607	0.020
rs1415263	*NOS1AP*	0.577	0.011
rs1966839	*DDC*	0.575	0.026
rs6704393	*NOS1AP*	0.417	0.052
rs17470454	*DTNBP1*	0.380	0.202
rs11133767	*DAT*	0.317	0.052
rs1858232	*NOS1AP*	0.306	0.078
rs9370823	*DTNBP1*	0.306	0.054
rs4646316	*COMT*	0.282	0.105
rs386231	*NOS1AP*	0.234	0.175
			Overall $P = 0.004539$

[1]Effect size is the coefficient of logistic regression between schizophrenia diagnosis and genotype code, expressed as the increase of log-odds per increase of genotype code (0 = homozygous a/a; 1 = heterozygous a/b; 2 = homozygous b/b).

Table 3: Linear regression between 50 SNPs and normalised cluster 1 scores.

SNP	Gene	Effect size[1]	*P*-value
rs2711844	*RELN*	0.046	0.003
rs410557	*PRODH*	0.045	0.001
rs4656355	*NOS1AP*	0.039	0.002
rs1966839	*DDC*	0.031	0.026
rs1049353	*CNR1*	0.024	0.078
			Overall $P = 0.000001$

[1]Effect size is the coefficient of linear regression between cluster 1 scores and genotype code, expressed as the increase of cluster score per increase of genotype code (0 = homozygous a/a; 1 = heterozygous a/b; 2 = homozygous b/b).

Table 4: Linear regression between 50 SNPs and normalised cluster 2 scores.

SNP	Gene	Effect size[1]	*P*-value
rs1468412	*GRM3*	0.130	0.013
rs450046	*PRODH*	0.130	0.015
rs2273816	*KPNA3*	0.101	0.009
rs2282956	*GRM3*	0.091	0.071
rs1415259	*NOS1AP*	0.090	0.061
rs6675281	*DISC1*	0.064	0.102
rs4531275	*NOS1AP*	0.057	0.072
rs1049353	*CNR1*	0.051	0.055
rs4656355	*NOS1AP*	0.050	0.005
rs2711844	*RELN*	0.047	0.138
			Overall $P = 0.001303$

[1]Effect size is the coefficient of linear regression between cluster 2 scores and genotype code, expressed as the increase of cluster score per increase of genotype code (0 = homozygous a/a; 1 = heterozygous a/b; 2 = homozygous b/b).

Table 5: Linear regression between 50 SNPs and normalised cluster 3 scores.

SNP	Gene	Effect size[1]	*P*-value
rs2734839	*DRD2*	0.044	0.186
rs386231	*NOS1AP*	0.044	0.057
rs1858232	*NOS1AP*	0.043	0.056
rs2282965	*GRM3*	0.043	0.048
rs6314	*HTR2A*	0.042	0.184
rs6347	*DAT*	0.041	0.081
rs4680	*COMT*	0.040	0.047
rs1966839	*DDC*	0.033	0.145
			Overall $P = 0.003342$

[1]Effect size is the coefficient of linear regression between cluster 3 scores and genotype code, expressed as the increase of cluster score per increase of genotype code (0 = homozygous a/a; 1 = heterozygous a/b; 2 = homozygous b/b).

Figure Legends

Figure 1: Dendrogram representing the clustering of forty four phenotypes.