A family-based association study of DNA sequence variants in GRM7 with schizophrenia in an Indonesian population

Clarissa Ganda, Sibylle G. Schwab, Nurmiati Amir, Heriani Heriani, Irman Irmansyah, Agung Kusumawardhani, Martina Nasrun, Ika Widyawati, Wolfgang Maier and Dieter B. Wildenauer

1 Western Australian Institute for Medical Research, Centre for Medical Research, University of Western Australia, Perth, WA, Australia
2 School of Psychiatry and Clinical Neurosciences, University of Western Australia, Perth, WA, Australia
3 School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia
4 Department of Psychiatry, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
5 Department of Psychiatry, University of Bonn, Bonn, Germany
6 Centre of Clinical Research in Neuropsychiatry (CCRN), Graylands Hospital, Mt Claremont, WA, Australia

Abstract

We previously reported genome-wide significant linkage to chromosome 3p in a sib-pair family sample from Indonesia. A promising candidate gene within the linked region is the metabotropic glutamate receptor subtype 7 (GRM7), involved in glutamatergic neurotransmission. We genotyped 18 single nucleotide polymorphisms in GRM7 in the sample of 124 Indonesian sib-pair families that had provided the significant linkage finding. Transmission disequilibrium analysis revealed nominally significant transmission distortion of rs17031835 in intron 1 of GRM7 (p = 0.004, before correction for multiple testing), along with haplotypes containing rs17031835. No other single marker was found to be significantly associated with schizophrenia in our sample. The results from our study provide support for the idea that glutamatergic neurotransmission and specifically the GRM7 gene might be relevant to the development of schizophrenia. Further studies supporting this finding are warranted.

Received 28 January 2009; Reviewed 24 April 2009; Revised 24 May 2009; Accepted 29 June 2009; First published online 29 July 2009

Key words: Family-based association, GRM7, Indonesia, schizophrenia.

Introduction

We recently reported genome-wide significant linkage to chromosome 3p26.2-25.3 [maximum likelihood score (MLS) = 3.76] with schizophrenia in 124 sib-pair families from Indonesia (Irmansyah et al. 2008). The broad linkage peak spans ~60 cM. A number of promising candidate susceptibility genes for schizophrenia are located in this area. Closest to the region with the highest LOD score (< 1 cM) is the gene encoding the metabotropic glutamate receptor subtype 7 (GRM7, mGluR7). The metabotropic glutamate receptors (mGluRs) represent one of the two classes of receptors mediating glutamatergic neurotransmission, the other being the ionotropic receptors comprising AMPA, NMDA and kainate. Dysfunction of the glutamatergic system has been hypothesized to contribute to the pathophysiology of schizophrenia. This hypothesis arose based upon observations of decreased levels of glutamate in the cerebrospinal fluid of schizophrenia patients (Kim et al. 1980). This finding was subsequently supported by studies demonstrating psychotomimetic effects of NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, in healthy individuals resembling the symptoms observed in schizophrenia patients (Javitt & Zukin, 1991; Krystal et al. 1994).

Currently, eight subtypes of mGluRs have been identified (mGluR1–8) and classified into three groups according to their sequence homology, signal transduction mechanisms and pharmacology. mGluR7 belongs to group III, together with mGluR4, -6, and -8.
This group of mGluRs is localized at presynaptic terminals, where they are thought to function as autoreceptors by regulating the feedback inhibition of glutamate release (Forsythe & Clements, 1990). Various studies of mGluR7-deficient mice have indicated putative roles in anxiety, emotional responding, and spatial working memory (Callaerts-Vegh et al. 2006).

Previous studies have investigated association of DNA sequence variants in the GRM7 gene with schizophrenia. Two earlier studies (Bolonna et al. 2001; Bray et al. 2000) investigated a single non-synonymous single nucleotide polymorphism (SNP) in exon 6 of the gene (rs2229902), which results in an amino-acid change (Tyr→Phe) of the encoded protein, in independent samples of European origin. Both studies failed to detect association of the variant with schizophrenia. More recently, however, Ohtsuki et al. (2008) conducted a more comprehensive study in which they tested 15 SNPs, within and flanking the gene, in a sample of 2293 Japanese patients and 2382 Japanese controls. This study detected association of a synonymous SNP in exon 1 of GRM7 (rs3749380) with schizophrenia (uncorrected \( p = 0.009 \), OR 1.12). In a recent study, Walsh and colleagues (2008) reported increased frequency of rare structural variants in patients with schizophrenia compared to unaffected controls. Genes disrupted by structural variants were found to be significantly over-represented in pathways important for brain development, including glutamate signalling. Specifically, a 136-kb microdeletion disrupting GRM7 was identified in a patient with schizophrenia.

The aim of the present study was to investigate whether DNA sequence variants in the GRM7 gene locus are associated with schizophrenia in our Indonesian sib-pair family sample with genome-wide significant linkage evidence to chromosome 3p.

Method

Ascertainment of families

Our sample comprised families from Indonesia with at least two affected offspring with clinically diagnosed schizophrenia or schizoaffective disorder. In most cases, DNA samples from both parents were available. However, in the case of missing parents, additional unaffected siblings were ascertained to facilitate family-based statistical analysis. For each individual, structured interviews were conducted by clinical psychiatrists using the following instruments: Diagnostic Interview for Psychosis (DIP; Jablensky et al. 2000), Family Interview for Genetic Studies (FIGS; Maxwell, 1992), Personal and Psychiatric History Schedule (PPHS; WHO, 1996), and the Operational Criteria Checklist for Psychotic Illness (OPCRIT; McGuffin et al. 1991). Consensus diagnosis was determined by at least two independent psychiatrists according to DSM-IV and ICD-10 criteria. The total sample consisted of 124 families comprising 540 individuals (267 affected individuals). A detailed description of our family sample can be found in Irmansyah et al. (2008). Each participating individual gave informed consent for participation in the study. The study was approved by the Institutional Review Board of the University of Indonesia and the University of Western Australia.

Genotyping

We originally selected 16 SNPs in the GRM7 gene to test for association with schizophrenia (Fig. 1a). This included all exonic SNPs with minor allele frequencies (MAFs) >0.05 (rs3749380, rs2229902, rs1485175, rs1485174). HapMap data of the GRM7 gene indicates the absence of large (>45 kb) linkage disequilibrium (LD) blocks in the region. Consequently, intronic markers were selected at regular intervals to provide even coverage of the gene (rs342026, rs17031835, rs7644436, rs339023, rs1154352, rs6804571, rs7609676, rs443102, rs712778, rs162723). Markers rs163422 and rs17047896 were included to provide some coverage of the 5’ and 3’ regions of the GRM7 gene. Following statistical analysis, we genotyped additional two markers (rs465152, rs339804) in high LD with the marker for which a nominally significant association was detected (rs17031835). Altogether, the 18 markers spanned 885.81 kb, providing an average inter-marker distance of 44.21 kb (with a range of 39–149 kb).

SNP genotyping was performed by high-throughput fluorescence-based allelic discrimination assays, such as TaqMan® SNP genotyping assays-by-demand (Applied Biosystems, USA) and Amplifluor® SNPs HT genotyping system (Millipore, USA), using previously described protocols (Morar et al. 2007). For three of the markers (rs17031835, rs7644436, rs712778), restriction fragment length polymorphism (RFLP) assays were also used in order to complete genotyping as well as to obtain confirmatory genotypes. All primers used for RFLP assays were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The restriction endonucleases used for rs17031835, rs7644436, and rs712778 were Aul, Hsp92II, and BslI, respectively.

Data analysis

Genotypic distributions of parental chromosomes were checked for deviations from Hardy–Weinberg
equilibrium (HWE) using \( \chi^2 \) test for goodness-of-fit (1 d.f.). Genotyping data was also checked for consistency with Mendelian inheritance. Association of single markers and haplotypes with schizophrenia was assessed using a modification of the transmission-disequilibrium test (TDT), as implemented in the statistical program FAMHAP (Knapp & Becker, 2003). This modification enables the TDT to be used as a valid test for association even in the presence of linkage and in families comprising more than one affected offspring. For testing haplotype associations, FAMHAP estimates haplotype frequencies using the expectation-maximization (EM) algorithm (Becker & Knapp, 2004).

HAPLOVIEW version 4.1 (Barrett et al., 2005) was used to evaluate the degree of LD between markers and for constructing haplotype blocks using the confidence interval algorithm of Gabriel et al. (2002). Finally, we corrected for multiple testing by SNP spectral decomposition (SNPSpD) analysis (Nyholt, 2004), which determines the number of independent markers in a given set and calculates the experiment-wide significance threshold required to keep type I error \( (\alpha) \) at 0.05.

**Results**

We genotyped a total of 18 SNP markers in a sample of 124 sib-pair families. A genotyping completion rate of > 98% was achieved for all markers except rs465152 (96.7%). Table 1 shows the allele frequencies of the 18 markers in our sample. As indicated, all markers had MAFs > 0.05. Marker genotype frequencies for the parental generation did not deviate significantly from HWE proportions \( (p > 0.05) \). Mendelian inheritance checks identified nine inconsistencies, involving seven SNPs and families, for which genotyping data was excluded from further analysis. Analysis of pair-wise LD and haplotype block structure of the original 16 SNPs in \( \text{GRM7} \) revealed weak LD \( (D' < 0.6) \) in the region, consistent with HapMap data (Fig. 1b). This observation was supported by SNPSpD analysis, which calculated 15 independent markers and an experiment-wide significance threshold of \( p = 0.003 \).

Single marker association analysis detected nominally significant over-transmission of the major allele \( (C) \) of rs17031835 to affected offspring \( (p = 0.004; \ T/NT = 28/10) \) (Table 1). No other markers were significantly
Table 1. Positions, inter-single nucleotide polymorphism (SNP) distances, allele frequencies, and \( p \) values for the 18 SNPs genotyped. Results from haplotype analysis of markers spanning rs342026 to rs339804 are also shown. Only marker combinations with nominally significant \( p \) values are given.

<table>
<thead>
<tr>
<th>SNP</th>
<th>dbSNP ID</th>
<th>Position (bp)</th>
<th>Inter-SNP distance (bp)</th>
<th>Alleles</th>
<th>Minor allele frequency</th>
<th>T/NT</th>
<th>( p ) value</th>
<th>Haplotype combinations (( p ) value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs163422</td>
<td>6875744</td>
<td>0</td>
<td>G/A</td>
<td>0.113</td>
<td>32/35</td>
<td>0.830</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>rs3749380</td>
<td>6878297</td>
<td>2553</td>
<td>C/T</td>
<td>0.385</td>
<td>58/76</td>
<td>0.109</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>rs342026</td>
<td>6883353</td>
<td>5238</td>
<td>C/T</td>
<td>0.481</td>
<td>71/83</td>
<td>0.461</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>rs465152</td>
<td>6894079</td>
<td>10544</td>
<td>A/G</td>
<td>0.171</td>
<td>51/52</td>
<td>0.852</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>rs17031833</td>
<td>6896758</td>
<td>2679</td>
<td>C/T</td>
<td>0.042</td>
<td>28/10</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>rs339804</td>
<td>6897197</td>
<td>439</td>
<td>A/G</td>
<td>0.167</td>
<td>54/56</td>
<td>0.792</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>rs7644436</td>
<td>6925189</td>
<td>27992</td>
<td>C/A</td>
<td>0.325</td>
<td>54/60</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>rs339023</td>
<td>6974311</td>
<td>49122</td>
<td>C/T</td>
<td>0.251</td>
<td>55/63</td>
<td>0.563</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>rs1154352</td>
<td>6988463</td>
<td>14152</td>
<td>G/A</td>
<td>0.119</td>
<td>33/35</td>
<td>0.907</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>rs6804571</td>
<td>7111588</td>
<td>123125</td>
<td>A/G</td>
<td>0.394</td>
<td>66/60</td>
<td>0.660</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>rs7609676</td>
<td>7222706</td>
<td>111118</td>
<td>C/G</td>
<td>0.395</td>
<td>63/65</td>
<td>0.929</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>rs6443102</td>
<td>7329957</td>
<td>107251</td>
<td>G/T</td>
<td>0.450</td>
<td>68/71</td>
<td>0.870</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>rs712778</td>
<td>7443858</td>
<td>113901</td>
<td>C/T</td>
<td>0.378</td>
<td>88/79</td>
<td>0.700</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>rs2229902</td>
<td>7469417</td>
<td>25559</td>
<td>A/T</td>
<td>0.107</td>
<td>33/30</td>
<td>0.822</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>rs1485175</td>
<td>7595789</td>
<td>126372</td>
<td>T/C</td>
<td>0.414</td>
<td>78/69</td>
<td>0.524</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>rs1485174</td>
<td>7595828</td>
<td>39</td>
<td>G/A</td>
<td>0.131</td>
<td>40/37</td>
<td>0.829</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>rs162723</td>
<td>7744798</td>
<td>148970</td>
<td>A/G</td>
<td>0.074</td>
<td>19/28</td>
<td>0.341</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>rs17047896</td>
<td>7761554</td>
<td>16756</td>
<td>A/G</td>
<td>0.074</td>
<td>17/26</td>
<td>0.308</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) NCBI Build 129.
\( ^b \) Major/minor allele.
\( ^c \) Transmission numbers are for the major allele.
\( ^d \) Haplotype frequency (affected/unaffected): 0.785/0.657.
\( ^e \) Haplotype frequency (affected/unaffected): 0.0790/0.674.
\( ^f \) Haplotype frequency (affected/unaffected): 0.783/0.657.
\( ^g \) Haplotype frequency (affected/unaffected): 0.497/0.407.
\( ^h \) Haplotype frequency (affected/unaffected): 0.495/0.402.
associated with schizophrenia in our sample, including the marker (rs3749380) previously reported to be associated. However, while not significant, our data did indicate preferential transmission of the minor allele (T) of rs3749380 to affected offspring (p = 0.109, T/NT = 76/58), which is consistent with the previous finding in a Japanese sample (Ohtsuki et al. 2008).

Whilst the observed association of rs17031835 did not reach significance, which was defined as p = 0.003 by SNPSpD analysis, we genotyped and tested an additional two markers (rs465152, rs339804) in high LD with rs17031835 for association with schizophrenia. We did not find evidence of association for these markers with schizophrenia in our sample (Table 1).

LD analysis confirmed LD between the three markers, which extends to include the upstream marker rs342026 (Fig. 1c). No other area showed a higher degree of LD in particular. Therefore, we restricted haplotype analysis to haplotype combinations comprising these four markers only. Five haplotype combinations produced nominally significant p values, ranging from 0.016 to 0.049 (Table 1). All significantly associated haplotypes contained the over-transmitted C allele of rs17031835.

Discussion

We estimated statistical power of our study using the Genetic Power Calculator (Purcell et al. 2003). Given a genotype relative risk (GRR) of 1.8, high-risk and marker allele frequencies of 0.2, and complete LD between alleles, study power was estimated to be 80% (a = 0.05). While a GRR of 1.8 may be considered too high for susceptibility variants contributing to schizophrenia, where expected effect sizes are typically in the range of 1.2–1.5, we argue that due to the strong linkage (MLS = 3.76) observed in our sample, it would be reasonable to expect enrichment of susceptibility variants of large effect. In contrast, however, the reported effect size for rs3749380 in the Japanese sample was much lower (OR 1.12) (Ohtsuki et al. 2008). Consequently, when GRR is <1.5, our study would not have had the power to detect association.

Whilst it is generally accepted that little variation in the prevalence and incidence of schizophrenia exists amongst different populations, studies have noted differences in terms of clinical presentation and outcome of schizophrenia in patients from developing countries as opposed to developed countries (Cohen et al. 2008; Sartorius et al. 1996). These differences may reflect variations in environmental influences, but just as likely, may reflect differing underlying genetic factors (genetic and/or allelic heterogeneity). Hence, genetic analysis of candidate genes in diverse populations can provide valuable information on susceptibility genes for schizophrenia.

The present study represents the first association study of GRM7 with schizophrenia in an Indonesian population. We selected the GRM7 gene for investigation based on its location in a region of chromosome 3p previously showing linkage to schizophrenia in our families. Whilst the broad linkage peak spans ~60 cM, the area of interest (flanked by microsatellite markers GATA131D09 and 295yc9P) spans ~5 Mb and contains 19 known genes. GRM7 is located in the region with the highest LOD score and given its known roles in glutamatergic neurotransmission we consider it a strong candidate susceptibility gene for schizophrenia. Other interesting candidate genes in this area have been previously described by us in Irmansyah et al. (2008), and include SLIT-ROBO Rho GTPase-activating protein 3 (SRGAP3) which plays a role in signal transduction. Here, we report weak evidence of association for an intronic marker in GRM7 with schizophrenia. Measures to validate this finding by testing two additional markers within the same haplotype block failed to confirm association. Hence, we cannot exclude the possibility that the reported association represents type I error, or a false-positive result. Alternatively, it is also possible that the associated marker is in LD with a risk variant present on a distinct haplotype block.

As is the case with most genetic association studies, our study relied on detecting risk variants through indirect association. That is, testing genetic markers in LD with the risk variant, rather than the risk variant itself, which is typically unknown. Such approaches are greatly facilitated by extensive LD blocks, as this dramatically reduces the number of markers that need to be genotyped. However, our data, along with HapMap data, indicates minimal LD in the region of GRM7. This lack of LD may have had a major impact on the outcome of our study, hampering our ability to detect association.

Historically, genome-wide linkage analysis has been widely applied to the search for genes conferring risk to human diseases. Whilst linkage analysis has been hugely successful in identifying the genetic basis of monogenic disorders, such as cystic fibrosis, its application to complex genetic disorders, such as schizophrenia, has yielded comparatively few successes. Nevertheless, linkage analysis has proved to be a useful tool in schizophrenia genetic research, directing our focus to a number of promising chromosomal regions, which have led to the identification of putative candidate genes. One such example is linkage to...
chromosome 8p and the subsequent identification of neuregulin 1 (NRG1) (Stefansson et al. 2002), one of the most well supported susceptibility genes for schizophrenia.

In summary, we previously reported genome-wide significant linkage to chromosome 3p in a family sample of Indonesian origin. To further investigate this finding, we tested variants in a positional candidate gene within this region for association with schizophrenia in our sample. The results from our study provide support for an involvement of the GRM7 gene in the aetiology of schizophrenia in the Indonesian population. However, the observed association of GRM7 was only weak and may account only in part for the linkage signal observed in our sample. Due to the lack of LD in the region of GRM7, future replication studies may benefit from analysing a denser set of markers for better coverage of the gene. Future studies would also be greatly facilitated by larger sample sizes in order to increase statistical power. As such, efforts are currently underway to collect a large case-control sample from Indonesia.

Acknowledgements

The authors thank all the individuals that participated in this study. Without their valuable contribution, none of this work would have been possible. Grant support for this work was provided by the National Health and Medical Research Council (NHMRC), grant no. 513861.

Statement of Interest

None.

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