Further evidence for genetic association of CACNA1C and schizophrenia: New risk loci in a Han Chinese population and a meta-analysis

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ABSTRACT

CACNA1C (12p13.3) has been implicated as a susceptibility gene for schizophrenia by several replicated genome wide association studies. While these results have been consistent among studies in European populations, the findings in East Asian populations have varied. To test whether CACNA1C is a risk gene for schizophrenia, we conducted a case-control study in 5897 schizophrenic patients and 6323 healthy control subjects selected from Han Chinese population. Our study replicated the positive associations of rs1006737 (P = 0.0108, OR = 1.16, 95% CI: 1.03–1.29) and rs1024582 (P = 0.0062, OR = 1.18, 95% CI: 1.05–1.33), and identified a novel risk locus, rs2007044 (P = 0.0053, OR = 1.08, 95% CI: 1.02–1.14). A meta-analysis of rs1006737 combining our study and previous studies was conducted in a total of 8222 schizophrenia cases and 24661 healthy controls. In the meta-analysis, the association between rs1006737 and schizophrenia remained significant (OR = 1.14, 95% CI: 1.07–1.22, P = 0.0001). Stratified analysis showed no heterogeneity between East Asian and European ancestries (χ²[1] = 0.07, P = 0.795), and the difference in pooled ORs between ancestries was not significant (Z = 0.25, P = 0.801). Our results provide further support for associations of rs1006737 and rs1024582 with schizophrenia, identify a new risk locus rs2007044 in a Han Chinese population, and further establish CACNA1C as an important susceptibility gene for the disease across world populations.

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1. Introduction

Schizophrenia is a debilitating disorder characterized by profound disturbances of cognition, emotion, and social functioning. It affects approximately 1% of the worldwide population (Andreasen, 1995; Bayer et al., 1999; Saha et al., 2005). To date, compelling evidence from family, twin, and adoption studies have indicated that many genes significantly contribute to the etiology of schizophrenia (McGuffin et al., 1995; Cannon et al., 1998; Owen et al., 2010). The disease’s heritability has been estimated to be between 80 and 85% (Sullivan et al., 2003). Despite such high heritability, the underlying genetic risk factors have yet to be identified.

One gene that has attracted much attention is the α1C subunit of the L-type voltage-gated calcium channel gene (CACNA1C, 12p13.3), which has been identified as a promising risk gene for schizophrenia by genome wide association studies (GWASs). In 2010, two large-scale studies conducted in European populations reported that allele A of rs1006737 was associated with risk for schizophrenia (Green et al., 2010; Nyegaard et al., 2010). A subsequent study performed by the Schizophrenia Psychiatric Genome-Wide Association Study Consortium reported that rs4765905, in complete linkage disequilibrium with rs1006737 (r² = 1), reached the genome-wide significant level (Ripke et al., 2011). This association was replicated in an extensive GWAS by Hamshere et al. (2013). Recently, another SNP at CACNA1C rs1024582,

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was demonstrated to be associated with the disease (Smoller et al., 2013). Notably, all of these SNPs are located within the same linkage disequilibrium block in an intronic region of CACNA1C, which may suggest that this gene region plays a role in conferring susceptibility to schizophrenia.

Even apparently highly significant positive findings require replication, preferably in several samples across a spectrum of ethnic groups. The first replication study performed on a non-European population, Hori et al., explored the association of rs1006737 and schizophrenia in a small set of Japanese subjects, but reported a negative result (Hori et al., 2012), that was inconsistent with the previous studies conducted in European populations. It is worth noting that both rs1006737 and rs4765905 have highly fluctuating minor allele frequencies (MAFs) among different ethnic groups (e.g. CEU: 0.347 vs. CHB: 0.067). MAFs that are below a certain threshold may create a detection limit that reduces a study’s power to detect true SNP associations. To address this issue, we decided to do a replication study to test whether CACNA1C is a risk gene for schizophrenia in the Han Chinese population using SNPs from the same genomic region reported in European populations. Even though previous association studies have yielded inconsistent results, we are not aware of any systematic meta-analysis of schizophrenia association study findings for CACNA1C. Therefore, to systematically evaluate the evidence for association and to enhance its potential power by identifying the source of inconsistent results, we also performed a meta-analysis combining our results with the findings of four previously published studies.

2. Methods

2.1. Subjects

Our study sample included 5897 unrelated schizophrenia patients (2831 males and 3066 females; mean age: 32.8 ± 7.1 years) and 6323 healthy controls (3098 males and 3225 females; mean age: 32.4 ± 8.3 years). All subjects were of Han Chinese ancestry from northern China. The consensus diagnoses were made by at least two experienced senior psychiatrists according to the Diagnostic and Statistic Manual of Mental Disorders, 4th edition (DSM-IV) criteria for schizophrenia. Patients with previously diagnosed diabetes, thyroid disease, hypertension, heart disease and other severe physical diseases were excluded. Healthy controls were recruited from communities with simple non-structured interviews performed by psychiatrists, who excluded individuals with any history of mental health and/or neurological disorder. Controls were matched with patients on location of their residence, gender and age. Written informed consent was obtained from all patients and their legal guardians and all healthy control subjects prior to the genetic study. The study was approved by the medical research ethics committees of the local hospitals and institutes from where patients and controls were recruited.

2.2. Genotyping

Peripheral blood samples were collected from all subjects. Genomic DNA was extracted from the blood using a Qiagen QIAamp DNA Mini Kit. Four SNPs spanning the genomic region that was previously reported to be associated with schizophrenia were selected from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), including rs2007044, rs1006737, rs882195 and rs1024582. Single SNPs were genotyped using TaqMan SNP genotyping assay on an ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, FosterCity, CA). PCR was performed following the standard protocol with 5 μl reaction volumes for each well in a 384-well plate and contained 5 ng of DNA. The thermal cycling conditions were 1 cycle at 95 °C for 10 min, 50 cycles of 92 °C for 15 s, and 60 °C for 1 min. The Sequence Detection System (SDS) Version 2.0 software (Applied Biosystems) was used for genotypic identification. For quality control purposes, all genotypes were blind to the case or control status during the genotyping process. We repeated the genotyping assay for 1% of the samples and found that the results were 100% concordant. The DNA extraction and genotyping were centrally processed at the Key Laboratory of Mental Health in Beijing.

2.3. Selection of literatures for inclusion and exclusion

We included case–control genetic association studies of CACNA1C in healthy controls and clinically diagnosed schizophrenia patients of any ethnic origin. Studies with data for only schizophrenia patients or only healthy participants were excluded, as were those whose authors did not respond to our data request. We searched PubMed, EMBASE and Medline for publications prior to May 31th, 2013, using the search terms: ‘schizophrenia’, ‘SCZ’, ‘CACNA1C’, ‘CACH2’, ‘CACN2’, ‘CACNL1A1’, ‘CCHL1A1’, ‘Cac1V1.2’, ‘MGC120730’, ‘TS’ and ‘rs1006737’. Once articles had been collected, bibliographies were hand-searched for additional references. Studies that reported previously published data were also excluded.

2.4. Data extraction

For each study, the following data were extracted independently by two authors (PFZ and WXX) using standard forms: (1) author(s) and year of publication; (2) methods (country of origin, dominant ancestry of sample, case and control sample size, diagnostic criteria for schizophrenia case, statement of Hardy–Weinberg equilibrium); and (3) data (number of participants in control and case groups, mean age and sex ratio by allele frequency). When the required data were not available in the published study or from web-based supplementary information, we contacted the authors of the paper by email to request access to the data. Genotype frequencies were used to calculate whether or not findings deviated significantly from Hardy–Weinberg equilibrium among controls. Subjects with either European or East Asian ancestry were coded.

2.5. Analysis of data

The statistical power of our sample size was calculated by Quanto 1.2.4 (Gauderman, 2002) (http://hydra.usc.edu/gxe/). Deviations in the genotype counts from the Hardy–Weinberg equilibrium were tested using a χ² goodness-of-fit test. Case–control association analysis was performed by SHEsis, a powerful software platform for analyses of LD, haplotype construction, and genetic association at polymorphism loci (Shi and He, 2005). Bonferroni correction for multiple testing was carried out to exclude type I errors.

For the meta-analysis, data were analyzed within a fixed-effect framework. Odds ratio (OR) was pooled using inverse variance methods to generate a summary OR and 95% confidence interval (CI). A fixed-effect framework assumes that the effect of allele frequency is constant across studies and between-study variation is considered to be caused by chance or random variation. We assessed the heterogeneity between included studies by the χ²-based Cochran’s Q statistic (Davey Smith and Egger, 1997; Egger et al., 1997). The percentage of variability across studies attributable to heterogeneity beyond chance was estimated using the I² statistic (Woodward, 2005). Stratified analysis was conducted to assess any moderating effect of ancestry (European and East Asian), Differences in pooled OR were compared using a Z test (Altman and Bland, 2003). Potential publication bias was assessed with the Egger’s test (Egger et al., 1997) and represented graphically with Begg’s funnel plots of the natural log of the OR versus its standard error (Begg and Mazumdar, 1994). A two-sided P value of less than 0.05 was judged significant for all analyses. All statistical meta-analyses were done with STATA (version 11; Stata Corp, College Station, TX).
3. Results

3.1. Case–control association results of CACNA1C with schizophrenia in Han Chinese population

We genotyped four CACNA1C SNPs in 5897 schizophrenia patients and 6323 healthy control subjects, all of whom were of Han Chinese ancestry. The genotyping call rate for all subjects was about 99.9%. Our sample size was sufficient to detect a significant difference at a power of about 90%, using OR values for the risk allele of 1.20 with a MAF of 0.05, as calculated by Quanto 1.2.4 using the additive model.

The genotype distributions for patients and controls were in Hardy–Weinberg equilibrium ($P > 0.05$). Allele frequencies between patients and controls are shown in Table 1. Three SNPs, including allele G of rs2007044 ($P = 0.0053$, OR = 1.081, 95% CI: 1.039–1.142), allele A of rs1006737 ($P = 0.0108$, OR = 1.156, 95% CI: 1.034–1.293) and allele T of rs1024582 ($P = 0.0062$, OR = 1.181, 95% CI: 1.048–1.330), were significantly associated with schizophrenia. After Bonferroni correction, they remained significant. The genotype distributions were also found to be statistically different between schizophrenia cases and healthy control subjects (Table S1).

3.2. Meta-analysis of rs1006737

3.2.1. Description of studies for meta-analysis of rs1006737

Five studies contributed to the meta-analysis of rs1006737, which contained data from a combined total of 6222 patients and 24,661 controls. The characteristics of these studies were described in Table 2.

3.2.2. Results of meta-analysis of rs1006737

When all samples were included, the association between rs1006737 allele frequency and schizophrenia case status was significant ($Z = 2.71$, $P = 0.007$, OR = 1.15, 95% CI: 1.04–1.27) and no evidence of significant between-study heterogeneity ($\chi^2 = 2$, $P = 0.10$, $I^2 = 0.0$). The association of rs1006737 allele frequency and schizophrenia case status was also significant when only samples from participants of European ancestry were included ($Z = 2.69$, $P = 0.007$, OR = 1.13, 95% CI: 1.03–1.24). There was no evidence of significant between-study heterogeneity in this sample either ($\chi^2 = 1$, $P = 0.991$, $I^2 = 0.0$). The difference in pooled ORs between East Asian and European samples was not significant ($Z = 0.25$, $P = 0.801$) and there was no heterogeneity between the two samples ($\chi^2 = 0.07$, $P = 0.795$). A detailed reproduction of these results is shown in Fig. 1.

A published imputation-driven meta-analysis reported rs1024582 was associated with schizophrenia (OR = 1.108, 95% CI: 1.057–1.163) (Smoller et al., 2013). We compared this OR with our result (OR = 1.181, 95% CI: 1.048–1.330) using the Z test, and found no significant difference ($Z = 1.11$, $P = 0.268$).

3.2.3. Stratified analysis for meta-analysis of rs1006737

When only samples from participants of East Asian ancestry were included, there was positive evidence for association between rs1006737 allele frequency and schizophrenia case status ($Z = 2.39$, $P = 0.01$, OR = 1.15, 95% CI: 1.04–1.27) and no evidence of significant between-study heterogeneity ($\chi^2 = 2$, $P = 0.10$, $I^2 = 0.0$). The association of rs1006737 allele frequency and schizophrenia case status was also significant when only samples from participants of European ancestry were included ($Z = 2.69$, $P = 0.007$, OR = 1.13, 95% CI: 1.03–1.24). There was no evidence of significant between-study heterogeneity in this sample either ($\chi^2 = 1$, $P = 0.991$, $I^2 = 0.0$). The difference in pooled ORs between East Asian and European samples was not significant ($Z = 0.25$, $P = 0.801$) and there was no heterogeneity between the two samples ($\chi^2 = 0.07$, $P = 0.795$). A detailed reproduction of these results is shown in Fig. 1.

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3.2.4. Publication bias for meta-analysis of rs1006737

The Begg’s funnel, presented in Fig. 2, did not indicate any evidence of publication bias. Neither Egger’s test ($t = 0.97$, $P = 0.402$) nor Begg’s test ($Z = 0.73$, $P = 0.462$) produced a statistically significant result.

4. Discussion

Confirming the findings of a GWAS or candidate gene study in another well-characterized population, especially across ethnicity, is an important and necessary step towards mapping susceptibility loci for complex psychiatric disorders such as schizophrenia. In the present study, we investigated the association of CACNA1C polymorphisms and schizophrenia with a relatively large sample from the Han Chinese population, including 5897 patients and 6323 healthy controls. Our findings did broadly replicate the association findings in European ancestry for previously reported CACNA1C loci rs1006737 and rs1024582, and obtain a positive signal with rs2007044, a nearby marker that has a similar MAF among different ethnic groups unlike the original positive marker rs1006737, which was highly polymorphic in European ancestry but less polymorphic in East Asian ancestry.

As expected, we found that rs1006737 and rs1024582 had much lower MAFs in our study group (Table 2). However, even with this low MAF, we detected positive associations between these SNPs and schizophrenia, which is consistent with the results of previous studies performed in European populations (Green et al., 2010; Nyegaard et al., 2010; Smoller et al., 2013). Considering the relatively small sample size of previous studies conducted in East Asian populations (Hori et al., 2012; Zhang et al., 2012), we concluded that the negative results might be type II error and should not be interpreted as contradictory to the previous literature linking CACNA1C to schizophrenia.

SNP rs4765905 was previously identified as a risk locus for schizophrenia by two GWASs (Ripke et al., 2011; Hamshere et al., 2013). Since rs4765905 is in complete linkage disequilibrium with rs1006737 ($r^2 = 1$), we did not test it in our study. Instead, we selected rs882195, a SNP located nearby, and tested its possible association with schizophrenia. Unfortunately, rs882195 showed no significant association with schizophrenia in our study.

So far, there is no absolute proof regarding the function of genetic variations at CACNA1C. Several studies have indicated that genetic variance at rs1006737 might be associated with changes in gene expression, brain structure, and function (Bigos et al., 2010; Krug et al., 2010; Wessa et al., 2010; Thimm et al., 2011; Wang et al., 2011; Zhang et al., 2012; Radua et al., 2013; Soeiro-de-Souza et al., 2013; Strohmaier et al., 2013). Although previous studies have investigated the functions of genetic variations at CACNA1C within an East Asian population (Hori et al., 2012; Zhang et al., 2012), they yielded contradictory results. One possible season for this could be that it is very difficult to recruit subjects with the genotypes needed for proper grouping due to the low MAF of reported positive markers in East Asian populations. The inclusion of the newly identified rs2007044 in our study eased this difficulty. While the current study firstly demonstrated a positive association between rs2007044 and schizophrenia up to now, further studies performed across various populations are needed to confirm this association before we can draw any conclusions related to the significance of this marker.

Table 1

Allele frequencies of four SNPs in CACNA1C gene between schizophrenia patients and controls in the Han Chinese population.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Position</th>
<th>Polymorphism</th>
<th>Associated allele</th>
<th>MAF (case = 5897)</th>
<th>MAF (control = 6323)</th>
<th>OR (95%CI)</th>
<th>$\chi^2$ (df = 1)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2007044</td>
<td>2215221</td>
<td>A/G</td>
<td>G</td>
<td>0.310</td>
<td>0.294</td>
<td>1.081</td>
<td>1.023–1.142</td>
<td>7.785</td>
</tr>
<tr>
<td>rs1006737</td>
<td>2215556</td>
<td>G/A</td>
<td>A</td>
<td>0.057</td>
<td>0.050</td>
<td>1.156</td>
<td>1.034–1.293</td>
<td>6.495</td>
</tr>
<tr>
<td>rs882195</td>
<td>2220662</td>
<td>C/G</td>
<td>G</td>
<td>0.386</td>
<td>0.377</td>
<td>1.038</td>
<td>0.958–1.093</td>
<td>9.599</td>
</tr>
<tr>
<td>rs1024582</td>
<td>2272507</td>
<td>C/T</td>
<td>T</td>
<td>0.051</td>
<td>0.043</td>
<td>1.032</td>
<td>1.005–1.061</td>
<td>1.330</td>
</tr>
</tbody>
</table>

* Polymorphism, the second allele is the minor allele in Asian populations as indicated in dbSNP database.

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First, there are considerable genetic differences between the Chinese and different ethnic populations. Among these differences, rs10024582 contributes equally to the genetic risk of schizophrenia even as MAF varies across populations, the risk allele of rs1006737 is associated with the risk for schizophrenia at a comparable power within both populations.

Considering that rs1006737’s MAF varies from 0.333 in European populations to 0.050 in Han Chinese populations, we initially thought that there must be heterogeneity between studies performed in these groups. However, our meta-analysis showed no heterogeneity between European and East Asian ancestries. To investigate whether heterogeneity exists between individual populations within the same ethnicity, we combined studies conducted in European and East Asian populations separately, but once again found no heterogeneity. Furthermore, we compared the OR of rs1024582 in our study with the reported OR (Smoller et al., 2013) but obtained no significant result.

Ethnic genetic heterogeneity is a common reason why studies detecting genetic polymorphisms that contribute to the susceptibility of complex human diseases are sometimes unable to be replicated. Our meta-analysis can partially help to resolve the inconsistent genetic association findings that have been published to date and revealed that, even as MAF varies across populations, the risk allele of rs1006737 and rs10024582 contributes equally to the genetic risk of schizophrenia among different ethnic populations.

There are, however, limitations to the interpretation of our results. First, there are considerable genetic differences between the Chinese and European ancestries. This fact is highlighted by the finding that SNPs rs1006737 and rs1024582 were common polymorphic variants in European sample sets, but were considerably less polymorphic in our sample set. Although meta-analysis and the comparison of ORs indicated that those two markers were stable risk loci among populations, the association of rs2007044 and schizophrenia still requires replication in another sample set, preferably across ancestry. Another possible limitation comes from our study’s sample size. A large sample such as ours is susceptible to population stratification. While this cannot be completely ruled out as an explanation for our positive association findings, it seems unlikely. Both our case and control samples were recruited from the same geographic area, northern China where residents have previously been demonstrated to form a homogenous cluster without observable substructure (Chen et al., 2009; Xu et al., 2009). Since controls were matched with patients on location of residence, gender and age, we might expect that if there was any subtle population admixture it would be similar in case and control subjects. Furthermore, we recruited samples by the same criteria that we used in a previous study (Yue et al., 2011), in which no obvious pattern suggesting population stratification was observed.

While our findings should be considered preliminary, they contribute important evidence for the establishment of CACNA1C as a susceptibility gene for schizophrenia across world populations. Further studies, including those using high density mapping and deep sequencing, are required to confirm our results and identify other common susceptibility loci. Future investigations should be especially focused on loci with similar MAFs in both East Asian and European ancestries, in order to verify the biological functions and pathogenic mechanisms of these genetic variations that are globally relevant.

In order to systematically evaluate the association between CACNA1C and schizophrenia, we also performed a meta-analysis combining previously published data with our new data. The overall meta-analysis supports the existence of a significant association between rs1006737 and schizophrenia. Our results show that, even though MAF differs greatly between European and East Asian ancestries, allele A of rs1006737 is associated with the risk for schizophrenia at a comparable power within both populations.

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<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Case Sample size</th>
<th>MAF</th>
<th>Control Sample size</th>
<th>MAF</th>
<th>Ancestry</th>
<th>Diagnosis</th>
<th>HWE</th>
<th>OR (associated allele)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green et al.</td>
<td>2010</td>
<td>479</td>
<td>0.355</td>
<td>15,316</td>
<td>0.327</td>
<td>European</td>
<td>DSM-IV</td>
<td>Yes</td>
<td>1.15 (A)</td>
<td>0.034</td>
</tr>
<tr>
<td>Nyeaard et al.</td>
<td>2010</td>
<td>976</td>
<td>0.361</td>
<td>1489</td>
<td>0.333</td>
<td>European</td>
<td>DSM-IV</td>
<td>Yes</td>
<td>1.16 (A)</td>
<td>0.015</td>
</tr>
<tr>
<td>Hori et al.</td>
<td>2012</td>
<td>552</td>
<td>0.067</td>
<td>1132</td>
<td>0.059</td>
<td>East Asian</td>
<td>DSM-IV</td>
<td>Yes</td>
<td>1.15 (A)</td>
<td>0.35</td>
</tr>
<tr>
<td>Zhang et al.</td>
<td>2012</td>
<td>318</td>
<td>0.061</td>
<td>401</td>
<td>0.057</td>
<td>East Asian</td>
<td>ICD-10</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Current</td>
<td>2013</td>
<td>5897</td>
<td>0.057</td>
<td>6323</td>
<td>0.050</td>
<td>East Asian</td>
<td>ICD-10</td>
<td>Yes</td>
<td>1.16 (A)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

* The original ORs and P values listed in papers.

**Fig. 1.** Meta-analysis of case–control studies of rs1006737 A allele frequency and schizophrenia case status. Meta-analysis indicates significant association between A allele frequency and schizophrenia (P < 0.001). Bars represent individual study 95% CI, with a central block proportional to study size. The summary diamond bar represents the pooled effect size.
Fig. 2. Funnel plot of effect size estimate (log OR) and S.E. of logOR. Possible publication bias would be evidenced by asymmetry in this plot. Symmetry in the plot in the predicted direction indicated no evidence of publication bias.

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Contributors
Fanfan Zheng, Yanling Zhang and Wuxiang Xie were responsible for the study design, statistical analysis, and manuscript preparation. Wenqiang Li, Chao Jin, Weifeng Mi, Fang Wang, Wenbin Ma, Cuicui Ma, Yongfeng Yang, Bo Du, Keping Li, Chenxing Lu, Lifang Wang, Tianlan Lu, Hongyan Zhang and Luxian Lv were responsible for recruiting the patients, performing the clinical rating and collecting the samples. Fanfan Zheng and Wuxiang Xie managed the literature searches and analyses. Yun Wang, Lin Lu, Dai Zhang and Weihua Yue were involved in evolving the ideas, writing the protocol, editing the manuscript and providing the funding for the study. All authors contributed to and have approved the final manuscript.

Conflict of interest
The authors declare no actual or potential conflict of interests in relation to this article exists.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.schres.2013.12.003.

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