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ORIGINAL INVESTIGATION

Association study of the *KCNJ3* gene as a susceptibility candidate for schizophrenia in the Chinese population

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Abstract We recently reported the results of a genome-wide association study (GWAS) of schizophrenia in the Japanese population. In that study, a single nucleotide polymorphism (SNP) (rs3106653) in the *KCNJ3* (potassium inwardly rectifying channel, subfamily J, member 3) gene located at 2q24.1 showed association with schizophrenia in two independent sample sets. KCNJ3, also termed GIRK1 or Kir3.1, is a member of the G protein-activated inwardly rectifying K^+ channel (GIRK) group. GIRKs are widely distributed in the brain and play an important role in regulating neural excitability through the activation of various G protein-coupled receptors. In this study, we set out to examine this association using a different population. We first performed a gene-centric association study of the *KCNJ3* gene, by genotyping 38 tagSNPs in the Chinese population. We detected nine SNPs that displayed significant association with schizophrenia (lowest $P = 0.0016$ for rs3106658, *Global significance* $= 0.036$). The initial marker SNP (rs3106653) examined in our prior GWAS in the Japanese population also showed nominally significant association in the Chinese population ($P = 0.028$). Next, we analyzed transcript levels in the dorsolateral prefrontal cortex of postmortem brains from patients with schizophrenia and bipolar disorder and from healthy controls, using real-time quantitative RT-PCR. We found significantly lower *KCNJ3* expression in postmortem brains from schizophrenic and bipolar patients compared with controls. These data suggest that the *KCNJ3* gene is genetically associated with schizophrenia in Asian populations and add further evidence to the "channelopathy theory of psychiatric illnesses".

Abbreviations

GWAS	Genome-wide association study
SNP	Single nucleotide polymorphism
KCNJ3	Potassium inwardly rectifying channel, subfamily J, member 3
GIRK	G protein-activated inwardly rectifying K^+ channel
GPCRs	G protein-coupled receptors
GABA	γ-Aminobutyric acid
PMI	Postmortem interval
LD	Linkage disequilibrium
HWE	Hardy–Weinberg equilibrium
SE	Standard error
NMDA	*N*-methyl-D-aspartate

Introduction

Schizophrenia is a highly heritable neurodevelopmental disorder, with a complex pathophysiology. It is thought that multiple susceptibility genes, each with a small effect, act in conjunction with epigenetic processes and environmental factors. Identifying these susceptibility genes is still challenging and the underlying mechanisms of disease remain largely elusive.

In a search for schizophrenia susceptibility genes, we recently carried out a whole genome association survey and replication study, analyzing two sets of samples from

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Japanese cohorts. This approach revealed novel candidate genes associated with schizophrenia in the Japanese population (Yamada et al. 2011). Among the top hit signals in the two-stage association analyses, the *KCNJ3* gene drew special attention because several other genome-wide association studies (GWASs) (O'Donovan et al. 2009; Tam et al. 2010; Williams et al. 2011) and gene expression studies (Arion and Lewis 2011; Bigos et al. 2010; Huffaker et al. 2009; Kalkman 2011) of schizophrenia and other psychiatric illnesses have revealed a potential role in disease etiology for ion channel genes. Genes for calcium channel [CACNA1C: calcium channel, voltage-dependent, L type, alpha 1C subunit], potassium channels [KCNE1: potassium voltage-gated channel, Isk-related family, member 1, KCNE2: potassium voltage-gated channel, Isk-related family, member 2, KCNH2: potassium voltage-gated channel, subfamily H (eag-related), member 2] and chloride transporters [NKCC1: sodium/potassium/chloride cotransporter 1, also known as SLC12A2 (solute carrier family 12, member 2), KCC2: potassium/chloride co-transporter 2, also known as SLC12A5 (solute carrier family 12, member 5)] have emerged from these studies. However, the role of the *KCNJ3* gene in schizophrenia pathogenesis has not been evaluated so far.

KCNJ3, also known as GIRK1/Kir3.1, belongs to the family of G protein-gated inwardly rectifying potassium (GIRK/Kir3) channels. GIRK channels play an important role in controlling neuronal excitability, by generating slow inhibitory potentials, following the activation of G protein-coupled receptors (GPCRs). These GPCRs, are in turn stimulated by various neurotransmitters, such as dopamine, serotonin, opioids and γ-aminobutyric acid (GABA). The involvement of these neurotransmitters and cognate GPCRs in schizophrenia has been suggested by multiple lines of evidence (Karam et al. 2010). GIRK channels are also implicated in the pathophysiology of several other diseases, such as epilepsy, addiction, Down's syndrome, ataxia and Parkinson's disease (Luscher and Slesinger 2010). Therefore, it is tempting to speculate that any of these systems related to GIRK channels may contribute to the psychiatric symptoms and cognitive defects seen in schizophrenia.

To corroborate the association of *KCNJ3* with schizophrenia in Asian populations, we examined the gene using the following approaches: (1) we performed gene-centric analyses using tag SNPs that span the entire *KCNJ3* gene region to achieve greater coverage of genetic variations; (2) we resequenced and analyzed the coding region of the gene; (3) we analyzed patient–parent pedigrees of Chinese descent, an ethnically similar but different population (Kim et al. 2005; Tian et al. 2008); (4) we performed an expression study of the gene using postmortem brains from patients with schizophrenia and bipolar disorder and from controls.

Materials and methods

Samples

Chinese samples consisted of 293 pedigrees (1,163 subjects: 9 trios and 284 quads) collected by the NIMH initiative (http://nimhgenetics.org/).

RNA from the dorsolateral prefrontal cortex (Brodmann's area 46) was obtained from the Stanley Medical Research Institute (http://sncid.stanleyresearch.org/) (Kim and Webster 2009, 2010). Brain samples were taken from 35 schizophrenics (26 males, 9 females; mean ± SD age, 42.6 ± 8.5 years; postmortem interval (PMI), 31.4 ± 15.5 h; brain pH, 6.5 ± 0.2), 35 bipolar disorder patients (17 males, 18 females; mean ± SD age, 45.3 ± 10.5 years; PMI, 37.9 ± 18.3 h; brain pH, 6.4 ± 0.3), and 35 controls (26 males, 9 females; mean ± SD age, 44.2 ± 7.6 years; PMI, 29.4 ± 12.9 h; brain pH, 6.6 ± 0.3). Diagnoses were made in accordance with DSM-IV criteria. There were no significant demographic differences between the schizophrenia, bipolar disorder, and control brains, in terms of age, PMI, and sample pH. All the patients with schizophrenia and bipolar disorder had previously received therapeutic drugs to treat their disease.

Gene-centric association study

SNP information was based on the UCSC database (http://genome.ucsc.edu/). Using ldSelect software (Carlson et al. 2004), tag SNPs were selected according to the following criteria: alleles captured with $r^2 \geq 0.8$ and minor allele frequency (MAF) $\geq$ 10% in the Han Chinese population. In the gene-centric association study, SNP genotyping was performed using the TaqMan system (Applied Biosystems, Foster City, CA, USA), following the manufacturer's recommendation. PCR was performed using an ABI 9700 thermocycler, and fluorescent signals were analyzed on an ABI 7900HT Fast real-time PCR System using Sequence Detection Software (SDS) v2.3 (Applied Biosystems).

Linkage disequilibrium (LD) between markers and departures from the assumption of Hardy–Weinberg equilibrium (HWE) were evaluated based on data from independent parents.

Brain tissue and quantitative RT-PCR

Real-time quantitative RT-PCR analysis was conducted using an ABI7900HT Fast Real-Time PCR System (Applied Biosystems). TaqMan probes and primers for *KCNJ3* and *GAPDH* (an internal control) were Assay-on-Demand™ or Assay-by-Design™ gene expression products (Applied Biosystems). All real-time quantitative RT-PCR reactions were performed in triplicate, based on the standard curve method.

Resequencing analysis of KCNJ3

The exons of *KCNJ3* were screened for genomic variants by direct sequencing of PCR products, using 58 unrelated Chinese schizophrenia samples. Information on the primers and conditions for amplification are available upon request. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and the ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). SNPs were detected using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analyses

The degree of LD between all SNP markers was evaluated and haplotype structures were constructed using HAPLOVIEW, version 4.2 (Barrett et al. 2005). Single SNP and haplotype association tests were conducted using UNPHASED, version 2.404 (Dudbridge 2008). For multiple testing corrections, the permutations were generated by randomizing the transmission status of the parental haplotypes. In each permutation, the minimum *P* value was compared to the minimum *P* value over all the analyses in the original data. This allows for multiple testing corrections over all tests performed in a run (Dudbridge 2008).

The Mann–Whitney *U* test (two-tailed) was used to evaluate changes in gene expression levels between control and disease groups.

Results

Association study of NIMH Chinese family samples

To examine association of the *KCNJ3* gene with schizophrenia in the Chinese population, we performed a family-based association study, by genotyping 293 pedigree samples (284 quad and 9 trio samples, consisting of 1,163 family members) of Chinese origin.

Based on the HapMap database (http://hapmap.ncbi.nlm.nih.gov/), 37 tagSNPs were selected and successfully genotyped in the Chinese pedigree sample (Table 1). The SNP, rs5835552, detected in our mutation screening was also genotyped. Therefore, we analyzed a total of 38 SNPs.

Single marker analysis revealed strong associations for rs3106658 ($P = 0.0016$) and rs3106651 ($P = 0.0019$) (Table 1). These SNPs are located in an intronic region approximately 1.7 and 12.4 kb downstream of exon 2 (Fig. 1). The initial marker rs3106653, which showed genetic association with disease in Japanese samples was also significant in the Chinese population ($P = 0.029$) (Table 1). The remaining six SNPs (rs3111037, rs1823002, rs2652443, rs4567888, rs6713287 and rs 1037091) showed nominally significant allelic association with disease (Table 1). Significant SNPs in the gene showed no

Table 1 SNP information

dbSNP[a]	Position[b]	Allele[c]	*P* value[d]
rs4571015	155545670	G/**T**	0.0522
rs3111037	155548559	A/**C**	***0.0339***
rs3106658	155568062	A/**G**	***0.0016***
rs11895478	155571123	C/**T**	0.0684
rs3106653	155575560	A/**C**	***0.0289***
rs3106652	155578422	**C**/T	0.2100
rs3106651	155578779	**C**/T	***0.0019***
rs11895336	155585934	**C**/T	0.2109
rs3113007	155592410	**A**/G	0.4855
rs12471193	155596137	A/**G**	0.1278
rs6711727	155596438	**A**/G	0.1374
rs1823002	155605132	A/**G**	***0.0413***
rs2652443	155605737	A/**G**	***0.0101***
rs2121085	155607465	**A**/G	0.0892
rs2121089	155609387	A/**C**	0.0561
rs1992701	155609638	**C**/T	0.4310
rs16838098	155612020	**A**/G	0.2591
rs2921436	155615006	C/**G**	0.1121
rs2921440	155616886	A/**G**	0.8137
rs4567888	155622752	**A**/T	***0.0312***
rs985092	155629561	**A**/G	0.3705
rs985535	155632935	A/**G**	1.0000
rs11899272	155634578	**C**/T	0.5525
rs6713287	155635168	A/**T**	***0.0280***
rs16838151	155636504	A/**G**	0.0956
rs13398937	155640347	C/**G**	0.5371
rs2053672	155643978	**C**/G	0.6419
rs12616121	155645682	A/**G**	0.8597
rs2591157	155648366	**A**/G	0.4245
rs1838674	155651541	A/**G**	0.1443
rs1037091	155652357	A/**G**	***0.0406***
rs1445654	155653558	A/**T**	0.2041
rs2961976	155654466	**G/T**	0.8658
rs2591152	155656952	**A**/C	0.4419
rs1445649	155682556	A/**G**	0.2632
rs2971902	155692378	**G**/T	0.1191
rs13030348	155695054	**C**/T	0.5076
rs5835552	155712135–155712136	(**-**/T)	0.4829

[a] Data taken from the NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/)

[b,c] The SNP positions and allele designations are from the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (dbSNP build 132) (http://genome.ucsc.edu/cgi-bin/hgGateway). The overtransmitted alleles are shown in bold. For rs2961976, both alleles were equally transmitted to the patients. For rs5835552, (–) allele was overtransmitted

[d] *P* values of <0.05 are indicated in bold and italic

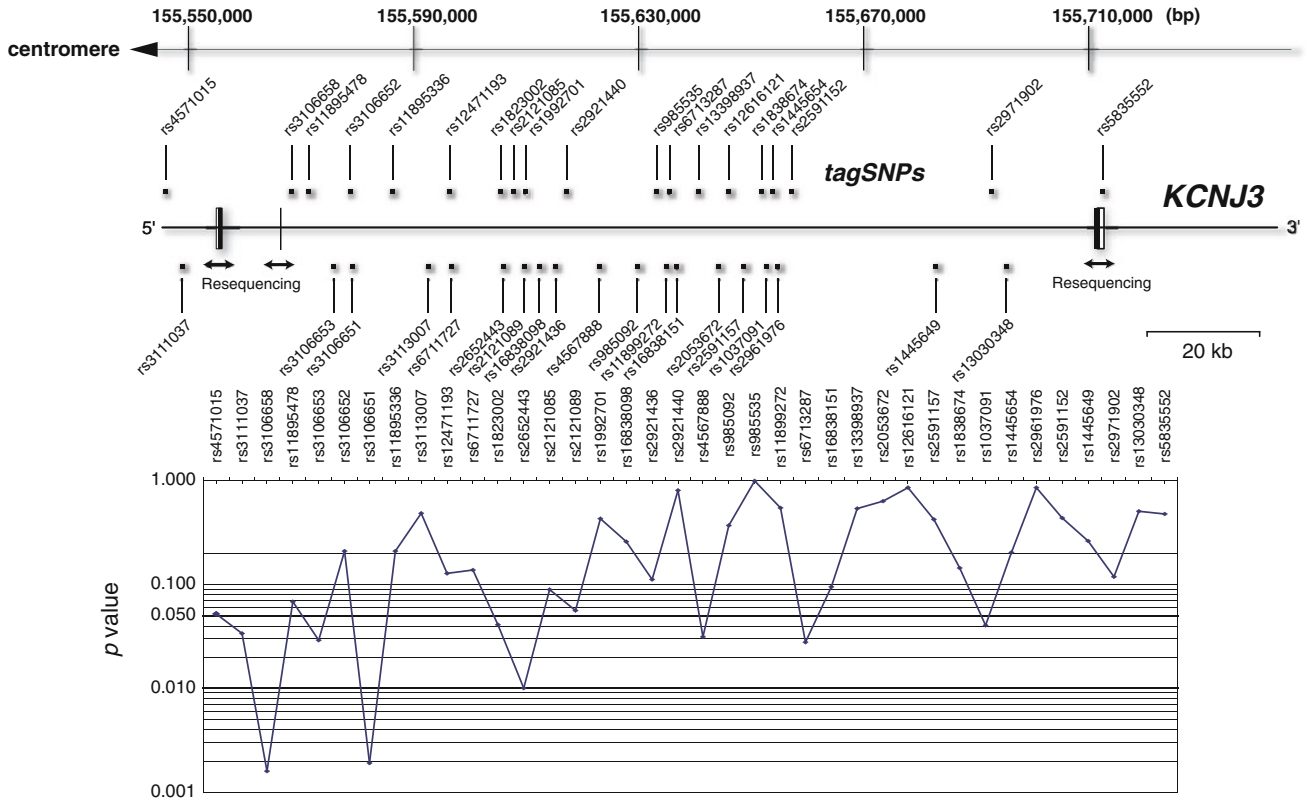

Fig. 1 Genomic structure of *KCNJ3* and gene-centric association analysis. In the *upper panel*, genomic structure and locations of examined tagSNPs in and around *KCNJ3* are shown, with chromosomal positions marked according to the human genome database (http://genome.ucsc.edu/) on the *top*. Gene exons are denoted by *boxes*. *White boxes* show non-coding regions. In the lower panel, the negative logarithm of *P* value for association is plotted as a function of chromosomal positions of SNP markers. *Horizontal bold line* indicates *P* value = 0.05 and 0.01

deviation from Hardy–Weinberg disequilibrium in the samples (independent parents of families).

Correction for multiple testing was conducted using the permutation method. This procedure gave a global significance level of $P = 0.036$ after 10,000 permutations [standard error (SE) = 0.00186].

Analysis of haplotype structures by the confidence interval method (Gabriel et al. 2002) identified eight haplotype blocks in the region (Fig. 2). We performed haplotypic tests for association (Supplementary Table S1). Three of the eight haplotype blocks showed significant association with disease (global $P = 0.0067$, 0.026 and 0.0386). The two most strongly associated SNPs (rs3106658 and rs3106651) were both located in haplotype block 2, however, most of the other significant SNPs were not in substantial LD to each other.

Real-time quantitative RT-PCR analysis in postmortem brains

The identification of *KCNJ3* as a susceptibility gene for schizophrenia led us to examine whether there was altered gene expression in the postmortem brains of patients. Real-time quantitative RT-PCR experiments showed that *KCNJ3* was down-regulated in the prefrontal cortex of schizophrenic and bipolar disorder patients, compared with controls (Fig. 3). To evaluate the effects of medication, Spearman rank-correlation coefficient was used to examine the relation between gene expression levels and a total lifetime exposure to the antipsychotic drug. The transcript levels were not correlated with lifetime use of antipsychotic drugs ($P = 0.647$ for schizophrenia, $P = 0.608$ for bipolar disorder patients).

Resequencing of the KCNJ3 gene

To screen for possible functional or causative variants of the *KCNJ3* gene, we resequenced all exons using 58 independent samples derived from schizophrenics. We found two novel and five known variants on or close to the upstream and downstream regions of the gene (Supplementary Fig. S1). No nonsynonymous SNPs were detected. Six of seven SNPs showed low allele frequencies (<6.2%) (Supplementary Fig. S1). Given the rarity of these variants, they were excluded from further analysis. The common SNP, rs5835552, was included in

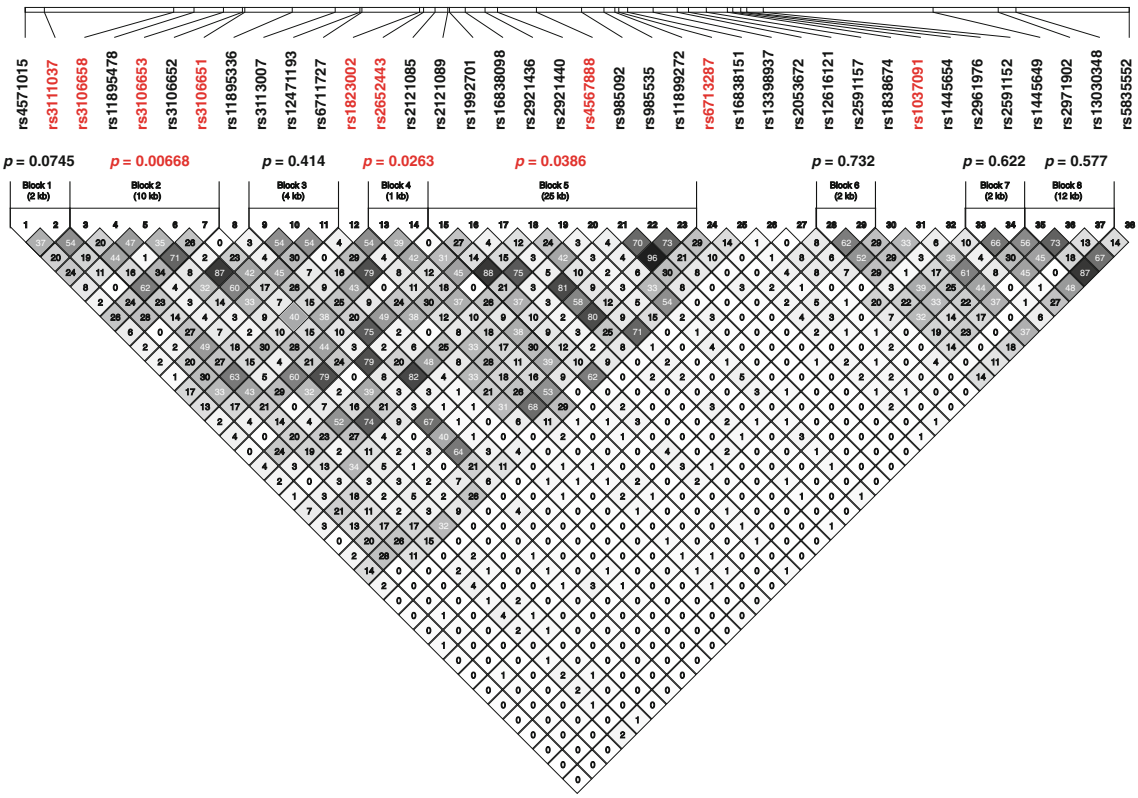

Fig. 2 Haplotype analysis of the *KCNJ3* gene. Haplotype structures were constructed using HAPLOVIEW, version 4.2 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). The global significance levels shown, were calculated using PDTPHASE implemented in the UNPHASED program version 2.404 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (Dudbridge 2008). Values in the *box* show the squared correlation coefficient (r^2) between the SNPs. Significant SNPs and haplotype blocks are shown in *red* ($P < 0.05$). Haplotype blocks 2, 4 and 5 showed significant haplotypic association with schizophrenia

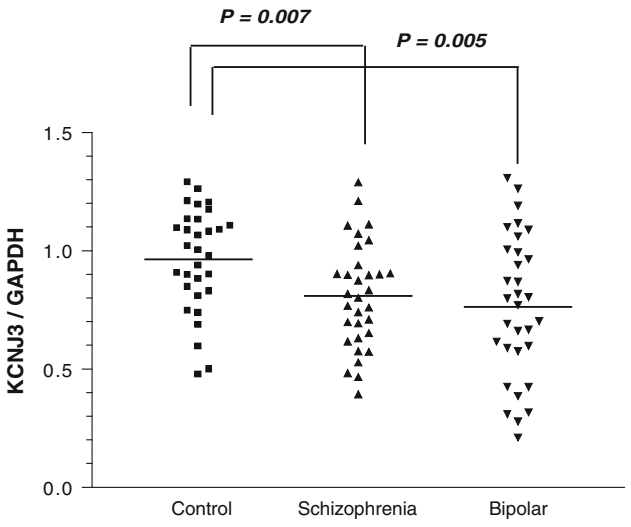

Fig. 3 Real-time quantitative RT-PCR analysis of the *KCNJ3* gene in postmortem brains. *Rectangles* and *triangles* represent individual samples. *Horizontal bars* delineate the mean of each group. The expression levels of *KCNJ3* were significantly decreased in the dorsolateral prefrontal cortex of postmortem brains from schizophrenics and bipolar disorder patients, compared with controls

the gene-based association study as stated above (not significant).

Discussion

In this study, we conducted a gene-based replication study for a novel candidate gene, *KCNJ3* identified from our prior GWAS in Japanese samples (Yamada et al. 2011), using a Han Chinese sample. We genotyped SNPs and resequenced exonic sequences to identify risk variants for schizophrenia. This approach allowed for greater coverage of genetic variations with the additional advantage of narrowing down regions relevant to functional variants, although in this case, we did not identify truly causative SNPs.

Single SNPs and haplotypic analyses have provided genetic evidence for the association of *KCNJ3* with schizophrenia in Chinese cohorts. Interestingly, the significant SNP markers and haplotypes were substantially not overlapped. This observation suggests a genetic evidence of allelic heterogeneity for disease risk spanning the

KCNJ3 gene. Furthermore, expression levels of *KCNJ3* were reduced in the prefrontal cortex of postmortem brains from schizophrenic and bipolar patients. We also performed genotyping of eight SNPs that are on the annotated genes among the top 20 signals (Yamada et al. 2011), however, these SNPs have failed to support association in Chinese population (Supplementary Table S2).

The *KCNJ3* gene is a novel candidate in schizophrenia genetics. The gene encodes a G protein-activated, inwardly rectifying potassium channel (GIRK1/Kir3.1) and belongs to the Kir3.x subfamily of inwardly rectifying potassium channels. Inwardly rectifying potassium channels are classified into seven subfamilies (Kir1.x to Kir7.x) and categorized into four functional groups (K^+ transport channels, classical Kir channels, G protein-activated K^+ channels and ATP-sensitive K^+ channels). Four genes, *KCNJ3, KCNJ6, KCNJ9* and *KCNJ5*, are included in the GIRK channel group (also termed *GIRK1–GIRK4* or *Kir3.1–Kir3.4*, respectively) (Hibino et al. 2010). GIRK channels play an important role in maintaining resting membrane potential and regulating the duration of action potentials (Hille 1992).

GIRK1 generally exist as heteromers with other GIRK subunits to form GIRK channels in native cells and tissues. The subunit composition of GIRK channels varies among different cells and tissues, allowing them to play diverse functional roles. In the central nervous system, neuronal GIRK channels are mostly heterotetramers of GIRK1 and GIRK2 subunits (Inanobe et al. 1999; Jelacic et al. 2000; Kofuji et al. 1995; Krapivinsky et al. 1995; Lesage et al. 1995; Liao et al. 1996; Yamada et al. 1998). The activation of GIRK channels by direct binding of the intracellular effector molecules, G$\beta\gamma$, results in channel opening. This channel opening hyperpolarizes cells, reducing neuronal excitability in many central neurons (Clapham and Neer 1993; Dascal 1997; Jan and Jan 1997; Logothetis et al. 2007; Sadja et al. 2003; Yamada et al. 1998). Many GPCRs in the central nervous system, including the receptors for somatostatin, 5-hydroxytryptamine (5HTR1), norepinephrine (ADRA2A, ADRA2B and ADRA2C), μ- (OPRM1), δ-opioid (OPRD1), dopamine (DRD2), glutamate (GRM1 to 8), cannabinoid (CNR1 and CNR2) and GABA (GABBR1 and 2) use signal transduction mechanisms through GIRK channels. These receptors are implicated in a variety of human disorders including schizophrenia (Karam et al. 2010).

These channels may contribute to a range of behaviors, anxiety, spasticity, pain and reward. GIRK1 and 2 knockout mice ($GIRK1^{-/-}$/$GIRK2^{-/-}$) are viable and appear normal, but they exhibit thermal hyperalgesia in the tail-flick test and display decreased analgesic responses, following intrathecal administration of morphine (Marker et al. 2004). In addition, $GIRK1^{-/-}$ mice display elevated open-field activity, decreased anxiety-like behavior, decreased baclofen ataxia and increased operant responding for food (Pravetoni and Wickman 2008).

Synaptic *N*-methyl-D-aspartate (NMDA) receptor activation in cultured hippocampal neurons increases surface expression of GIRK1 and GIRK2 subunits in the soma, dendrites, and dendritic spines (Chung et al. 2009a, b). Conversely, NMDA receptor antagonists inhibit GIRK complex formation (Kobayashi et al. 2006). In this study, real-time quantitative RT-PCR experiments showed that *KCNJ3* is down-regulated in the prefrontal cortex of schizophrenic patients. This finding may lend support to the "hypo-NMDA theory of schizophrenia". Recent studies suggest a genetic association between neuregulin-erbB receptor signaling and schizophrenia (Pitcher et al. 2011; Stefansson et al. 2002), and a functional link between neuregulin-erbB receptor signaling and GIRK is also reported (Ford et al. 2003).

Genetic studies raise the possibility that schizophrenia shares genetic risk factors with other psychiatric disorders (Cascella et al. 2009; Crespi et al. 2010; Rzhetsky et al. 2007). In this study, decreased gene expression was observed in the brains from bipolar disorder, as well as schizophrenia patients. For mood disorders, chronic administration of the selective serotonin reuptake inhibitor, fluoxetine, exerts a beneficial effect on a rodent model of depression, through suppression of GIRK-dependent signaling (Cornelisse et al. 2007). In addition, acute inhibition of GIRK channels by fluoxetine causes substantial suppression of neuronal cell death in mice with mutant GIRK channels (Takahashi et al. 2006). Interestingly, the *KCNJ3* gene lies in one of only two regions to have achieved genome-wide significance for linkage in autism (Consortium IMGSoA 2001). Recently, molecular cytogenetic characterization of two unrelated patients with 2q deletions, both of whom are affected by developmental delays with communication impairment, and one of whom has a diagnosis of autistic spectrum disorder, revealed that *KCNJ3* is the only gene located within the overlapping region of the two deletions (Newbury et al. 2009). In epilepsy, SNP, rs17642086 (1038T>C, His346His) in *KCNJ3* shows positive association with idiopathic generalized epilepsy ($P = 0.0097$) (Chioza et al. 2002). However, this SNP was in weak LD with our significant SNPs on *KCNJ3*, and we found no genetic association between this SNP and our schizophrenia samples ($P = 0.816$).

Copy number variants (CNVs) are emerging as an important genomic cause of neuropsychiatric disorders (Coyle 2009). In addition, altered microRNA (miRNA) expression profiles are suggested in the pathogenesis of schizophrenia (Merikangas et al. 2009; Stankiewicz and Lupski 2010). The region does not contain any known CNVs according to a database (Database of Genomic Variants: http://projects.tcag.ca/variation/). By contrast,

five conserved sites for miRNA families are detected by the TagetScanHuman (Release 5.2: June 2011, http://www.targetscan.org/) and there are 131 potential miRNA target sites in the gene region (miRNAMAP: http://mirnamap.mbc.nctu.edu.tw/). Therefore, the roles of miRNAs in the gene expression levels need further scrutiny.

In conclusion, we have provided evidence that *KCNJ3* contributes to schizophrenia in Asian populations, adding another susceptibility candidate gene to the "channelopathy theory of psychiatric illnesses". Our findings suggest a significant but a modest association between schizophrenia and the *KCNJ3* gene. Thus, future studies with a much larger sample size in Asian and/or in other ethnicities are needed to confirm the genetic contribution of the gene for schizophrenia. Moreover, genetic studies on channel genes including genes in potassium channels are warranted to improve the understanding of schizophrenia pathogenesis.

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Conflict of interest None.

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