Genetic association and identification of a functional SNP at GSK3β for schizophrenia susceptibility

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ARTICLE INFO

Article history:
Received 9 May 2011
Received in revised form 26 August 2011
Accepted 17 September 2011
Available online xxxx

Keywords:
GSK3β
Schizophrenia
Association
Han Chinese
EMSA
Reporter gene assay

Abstract

Objective: GSK3β is a key gene in neurodevelopment, and also an important target of antipsychotics. Several lines of evidence including association and gene expression studies have suggested GSK3β as a susceptibility gene for schizophrenia, but the underlying genetic mechanism is still unknown. In this study, we test whether the genetic variants in GSK3β contribute to the risk of schizophrenia in Chinese population.

Methods: We first conducted an association analysis of 9 representative SNPs spanning the entire genomic region of GSK3β in two independent Han Chinese case–control samples from southwestern China (the Kunming sample and the Yuxi sample, a total of 2550 subjects). Then using EMSA and reporter gene assays, we tested the functional impact of the identified risk SNP on transcriptional factor binding affinity and promoter activity.

Results: We observed weak allelic associations of three GSK3β SNPs (rs3755557, rs7431209 and rs13320980) with schizophrenia in the combined Han Chinese samples. Further analysis using genotypes (under recessive genetic model) supported the association of rs3755557 (p = 0.01, corrected), which is located in the GSK3β promoter region. The functional assays demonstrated that the risk SNP (rs3755557) could influence the transcription factor binding affinities, resulting in a higher promoter activity of the risk allele.

Conclusion: Our findings suggest that GSK3β is likely a risk gene for schizophrenia, and its expression alteration caused by the risk SNP in the promoter region may contribute to the etiology of schizophrenia.

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1. Introduction

Schizophrenia (MIM181500) is one of the most severe mental disorders with worldwide lifetime prevalence approaching 1%, and characterized by psychotic features (delusions and hallucinations), disorganization, dysfunction in normal affective responses, and altered cognitive function (Mueser and McGurk, 2004). It has been demonstrated that genetic component plays an important role in the etiology of schizophrenia, but the underlying genetic risk factors are yet to be identified.

The currently prevailing hypothesis has proposed that schizophrenia is a neurodevelopmental disorder, and disturbances in the early stage of brain development may cause abnormal alterations during embryonic neurogenesis, including regulating neuronal progenitor proliferation (Mao et al., 2009), changing synaptic morphology and physiology, altering differentiation, migration and survival of neurons, etc., which may result in impaired neuronal network in the mature brain and eventually lead to schizophrenia (Fatemi and Folsom, 2009).

The GSK3β gene, spanning about 286 kb on human chromosome 3q13.33 with 12 exons, is highly expressed in the brain and plays numerous roles in development, including synaptic plasticity modulation, intracellular trafficking, apoptosis and regulation of gene transcription etc. (Hur and Zhou, 2010). GSK3β is a key player in the Wnt and PI3K/AKT signaling pathways, which are crucially involved in neurodevelopment and also show abnormalities in schizophrenia (Cotter et al., 1998; Emamian et al., 2004; Lie et al., 2005). It has been suggested that GSK-3β is a master regulator of neural progenitor homeostasis, supporting the pivotal role of GSK-3β in brain development and function (Kim et al., 2009). Additionally, there are abundant evidences that GSK3β interacts with many known schizophrenia risk genes during neurodevelopment, implying potential involvement of GSK3β in schizophrenia susceptibility (Figure S1).

The involvement of GSK3β in schizophrenia are also supported by previous association studies, in which several markers have been identified, though the results were inconsistent among different
studies (Scassellati et al., 2004; Ikeda et al., 2005; Lee et al., 2006; Szczepankiewicz et al., 2006; Meng et al., 2008; Souza et al., 2008). In addition, the postmortem studies indicated that GSK3β may play an important role in the pathogenesis of schizophrenia, and a reduction of approximately 40% in GSK3β protein, mRNA and kinase activity was reported in the postmortem frontal cortex of schizophrenia patients (Kozlovsky et al., 2000, 2001, 2004, 2005). However, there have been inconsistent reports (Nadri et al., 2004). Collectively, whether GSK3β contributes to schizophrenia susceptibility awaits further studies.

To search for the susceptible genetic variants of GSK3β for schizophrenia, we first conducted a two stage case–control association analysis using samples independently collected from two cities (Kunming and Yuxi) of southwestern China (a total of 2550 subjects), and we identified a risk SNP (rs3755557) which is located in the GSK3β promoter region. With the use of EMSA and reporter gene assays, we proved that rs3755557 is a functional polymorphism.

2. Materials and methods

2.1. Subjects

We recruited two case–control samples independently from Kunming (432 cases, mean age 35.5±9.8 years and 568 controls, mean age 36.1±6.8 years) and Yuxi (520 cases, mean age 38.6±10.4 years and 1030 controls, mean age 36.7±6.9 years) of southwestern China. The patients were diagnosed with schizophrenia according to The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria (Kunming) and The International Classification of Diseases 10 (ICD-10) (Yuxi) respectively. Standard diagnostic assessments were supplemented with clinical information obtained by a review of medical records and interviews with family informants. Detailed information on the onset and course of clinical disorders, symptoms such as delusions and hallucinations, lack of motivation and social withdrawal etc. were obtained. All available information (personal history, hospital record and family history report) was also recorded. Potential patient participants who have history of alcoholism, epilepsy, neurological diseases, drug abuse, or other symptomatic psychoses were excluded from the present study. Meanwhile, unrelated healthy volunteers were recruited from the local communities as control subjects. These control individuals were all asked to provide detailed information about medical and family psychiatric histories. Those people who had history of mental disorders, psychiatric treatment, alcohol dependence, drug abuse, or family history of psychiatric disorders were excluded.

All the patient and control subjects were of Han Chinese origin from Yunnan province of southwestern China. All individuals were provided with written informed consents for participation, and the research protocol was approved by the internal review board of Kunming Institute of Zoology, Chinese Academy of Sciences.

2.2. SNP selection

For the initial screening in the Kunming sample, a total of 9 SNPs were selected. We first examined the LD pattern of GSK3β gene in the CHB population (Han Chinese from Beijing) using data from HapMap, and selected 8 tagging SNPs (rs3755557, rs334558, rs6774210, rs7431209, rs6872799, rs13320980, rs16830594 and rs4688043) with the use of the tagger procedure implemented in Haploview (Barrett et al., 2005). Two of these eight SNPs are located in the GSK3β promoter region (rs334558 and rs3755557) and the other SNPs are all located in the intron regions. We also selected another intronic SNP (rs6438552) which was proven to influence the alternative splicing of GSK3β gene (Kwok et al., 2005). All the selected SNPs are biallelic. Totally, 9 SNPs were selected for genotyping in the Kunming sample. For the replication analysis in the Yuxi sample, the most strongly associated SNPs in the Kunming sample were selected (rs3755557, rs7431209 and rs13320980). The genomic structure of GSK3β gene and the locations of the tested SNPs are shown in Fig. 1. The LD map of the tested GSK3β SNPs in the Kunming sample is shown in Fig. 2, and the SNP information is shown in Table 1.

2.3. SNP genotyping

Venous blood was collected from all participants. Genomic DNA was extracted from the blood sample using the standard phenol-chloroform method. DNA samples of the cases and controls were randomly distributed in the DNA plates. The primers were designed to amplify the regions containing the selected SNPs (length of PCR product = 1000 bp). Details of all primers and assay conditions are available on request. The PCR reactions were performed in the 96-well plates with a total volume of 25 μl, including 10 ng of genomic DNA. Genotyping was conducted using SNPShot method on an ABI 3130 automatic sequencer, which has already been described in our previous study (Luo et al., 2008). The genotypes of the tested SNPs were automatically called by ABI GeneMapper 4.0 and verified manually, and any assay with low quality was PCR amplified and genotyped again. To make sure of the accuracy of genotyping, we used bi-directional sequencing on randomly selected 120 individuals and no genotyping errors were found. The genotyping success rate for the 9 tested SNPs was more than 99.0%.

2.4. Electrophoretic mobility shift assay (EMSA)

For the functional prediction of the candidate SNP, we used a web-based program Alibaba (http://www.gene-regulation.com/pub/programs.html) to analyze potential protein binding sequences containing SNP rs3755557.

EMSAs were performed using gel shift assay systems (Pierce) under the provided guidelines. The single-strand oligonucleotides were 3’-end Biotin labeled and then annealed to form double strands. The binding reactions contained 10× binding buffer, 4 μg nuclear extract proteins, poly (dl–dc), and, if needed, unlabeled competitors; labeled probes were added after 20 min, and samples were incubated at room temperature for a total of 30 min. For antibody supershift assays, nuclear extracts were incubated with the labeled probe for 30 min at room temperature before the addition of TBP antibody. After adding the TBP antibody, the binding reaction was incubated for an additional 16 h at 4 °C. After incubation, samples were separated on a native 6% polyacrylamide gel and then transferred to a nylon membrane. The positions of biotin end-labeled oligonucleotides were detected by a chemiluminescent reaction with streptavidin–horseradish peroxidase according to the manufacturer’s instructions and visualized by autoradiography. The nucleotide sequences of the double-stranded oligonucleotides with either A or T allele are:

- A-allele: 5’-TGTCTCCAGAAAGGACTGTTAAAGGACCTATGGTA-TCA-3’
- T-allele: 5’-TGTCTCCAGAAAGGACTGTTAAAGGACCTATTTGTA-TCA-3’

2.5. Promoter cloning and reporter gene assays

In order to compare the promoter activities of the two alleles at rs3755557, individual homozygotes with respect to the corresponding genotypes (TT and AA) for rs3755557 were amplified encompassing nucleotides −1779 to +98 (relative to the transcription start site +1) of the GSK3β gene. Then the amplified fragments were cloned into the pGL3-basic plasmid vectors. We verified all recombinant clones by bidirectional DNA sequencing to make sure no de novo mutation was introduced. These plasmids were all accurately quantified by Biophotometer (Eppendorf) and equal amount of the plasmids were used for transfection. The pGL3-basic plasmid with a cloned promoter fragment

containing either T-allele or A-allele was transiently co-transfected into Human HEK293T and HeLa cell lines with pRL-TK plasmid (internal control) using Lipofectamine 2000 transfection reagent (Invitrogen). After 36 h incubation, we collected the cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). All assays were performed in at least three independent experiments with minimum of five replications.

2.6. Statistical analysis

The Haploview program (version 4.1) was applied to test the genotypic distributions of SNPs for Hardy–Weinberg equilibrium (HWE), to estimate linkage disequilibrium (LD) between paired SNPs using the \( r^2 \) algorithm, and to determine the haplotype blocks (Barrett et al., 2005). Allelic and genotypic associations were accessed with PLINK, and the results were corrected by Bonferroni method based on the total number of tests conducted (36 tests in total considering the 9 SNPs and four types of genetic models for allelic, additive, dominant and recessive tests) (Purcell et al., 2007). Haplotype-based association analysis was conducted using Haplo.Stats Package in R environment and only those haplotypes with a frequency of \( \geq 0.01 \) in the control subjects were considered. Corrections for multiple haplotypes and global analysis were performed with 20,000 permutations (Schaid et al., 2002). Power analysis was performed using the software G’power (Faul et al., 2007). The commonly used effect size of 0.1 and 0.2 were used in the power analysis, which are correspondent with a commonly used effect size of 0.1 and 0.2 were used in the power analysis. The other 6 SNPs did not show significance, and the results were shown in Table S2. We then examined the haplotypes incorporating the 3 SNPs (rs3755557, rs7431209, and rs13320980) in both Kunming and Yuxi samples. For the 3-SNPs haplotype analysis, we observed two risk haplotypes (A-G-T, \( p = 0.02, 15.9\% \) in cases versus 11.3\% in controls, and T-G-C, \( p = 0.001, 7.5\% \) in cases versus 4.0\% in controls) significantly associated with schizophrenia in the Kunming sample, and the global test also supported the associations (global \( p = 9.0 \times 10^{-4} \)). However, these associations were not replicated in the Yuxi sample (Table S3). We also examined the 2-SNPs and 9-SNPs haplotypes, and similar results were obtained (Table S4–S6).

To further test the association of GSK3β with schizophrenia, we performed genotypic association analysis under the dominant and recessive genetic models respectively. The results indicated that the frequency of AA-genotype for rs3755557 was higher in schizophrenia cases than in controls in both Kunming and Yuxi samples though did not remain significant after correction (\( p = 0.007 \) and \( p = 0.017 \), respectively, uncorrected). When the two samples were combined, the significance was strengthened (\( p = 0.0003 \), uncorrected), and still significant after Bonferroni correction (\( p = 0.01 \), corrected) (Table 2). No genotypic association was detected for rs3755557 under the dominant genetic model (Table S7). Similar results were obtained for rs13320980 sample (\( p = 0.01 \)). We double checked the eletromorphs of the original genotype data and no calling errors were found (Figure S2).

Table 1 presents the results of allelic association. For the Kunming sample, among the 9 tested SNPs, one SNP (rs13320980) showed nominally significant association with schizophrenia (\( p = 0.002 \), uncorrected). There are two SNPs with marginal significance (\( p = 0.050 \) for rs7431209 and \( p = 0.096 \) for rs3755557, uncorrected). For the Yuxi sample, however, we did not replicate the associations, though the tendency was in the same direction. When we combined the two samples, the association of rs13320980 remained nominally significant (\( p = 0.005 \), uncorrected). The significances for the other two SNPs were strengthened in the combined sample (\( p = 0.04 \) for rs7431209 and \( p = 0.03 \) for rs3755557, uncorrected) (Table 1), but none of them survived the Bonferroni corrections. Similar results were observed when conducting genotypic association analysis under the additive genetic model (Table S1). The other 6 SNPs did not show significant association and the results were shown in Table S2.

Fig. 1. The genomic structure of GSK3β and the physical locations of the 9 tested SNPs.

Fig. 2. The LD maps of the 9 tested GSK3β SNPs in the Kunming sample. The linkage disequilibrium (LD) of the tested SNPs was calculated using the \( r^2 \) algorithm in the Haploview program.

and rs7431209, in which the genotypic associations were stronger under the recessive genetic model compared with those under the dominant genetic model (Table S8 and Table S9).

We performed a power calculation using the G* power program based on Cohen’s method (Cohen, 1988). The present sample size of Kunming and Yuxi both revealed >81% and >95% power of detecting a significant association with allele and genotype (β < 0.05) given an effect size index of 0.1 (corresponding to a “weak” gene effect). When an effect size index of 0.2 was assumed, i.e. a “weak to moderate” gene effect, the sample size of Kunming and Yuxi both showed >97% power to detect significance (β < 0.05).

### 3.2. Electrophoretic mobility shift assays

To evaluate allele-specific effects on binding affinity of transcription factors, we performed EMSA analysis. The binding reactions were done with two identical probes differing only in respect to rs3755557 alternative alleles (A/T). The result showed that the predicted binding sequence containing the A-allele of rs3755557 could bind the nuclear extracts (a mix of nuclear proteins extracted from HEK293T cells), but the T-allele could not (Fig. 3, Lane 2–4). The addition of 200× molar excess of unlabelled probes with the same alleles could compete for protein binding, indicating that the nuclear extracts bind specifically to the sequence motif containing rs3755557 (Fig. 3, Lane 4–5). In addition, the binding was highly specific for the A-allele as reflected by the unaffected binding affinity using the cross competitor with 100× and 200× molar excess unlabeled probe of the T-allele (Fig. 3, Lane 6–9). Collectively, the EMSA assay suggested an on-and-off effect of the two alleles at rs3755557 on binding transcriptional factors.

### 3.3. In vitro promoter assay

To test the effect of rs3755557 on promoter activity, we cloned a DNA fragment of human GSK3β promoter, containing either the A allele or the T allele at rs3755557, and then transiently transfected it into human HEK293T and HeLa cell lines. The relative transcriptional activities were compared between the two alleles using luciferase reporter gene assay. As expected, the transcriptional activity of the GSK3β promoter containing the A allele is significantly higher than that of the T allele in both HEK293T (p = 0.001, Fig. 4a) and HeLa cells.
Potential susceptibility loci for schizophrenia on chromosome 3q was detected by early genome-wide analysis using microsatellite markers, which might suffer from the inherent heterogeneity commonly seen in postmortem samples. Second, the reduction of GSK3β expression was only reported in the frontal cortex, and whether this was true for the other brain regions are yet to be tested. Third, as GSK3β is the target of antipsychotics, which could inhibit GSK3β activities, the reported decrease of GSK3β expression in schizophrenia patients might be an artifact due to drug treatment. Finally, given the reduced expression in schizophrenia patients is true, this could also be caused by the genetic variants of other genes interacting with GSK3β in the schizophrenia-related pathways (e.g. Akt1 and DRD2). Additionally, given the reported lower protein amount in the post-mortem brains of schizophrenia patients, it is also possible that the unknown transcription factor(s) acting on the sequence containing rs3755557 can be inhibitory.

On the other hand, there has also been data suggesting increased GSK3β activity in schizophrenia patients. One study has reported that there are Akt1 deficits in schizophrenia patients, and the dysfunction of this inhibitory regulator for GSK3β can lead to hyper-activation of GSK3β (Emamian et al., 2004). Data from transgenic mice also suggested that the mice carrying a constitutively active mutant form of GSK3β displayed hyperactive behaviors and mania, which is similar with the symptoms of schizophrenia patients (Prickaerts et al., 2006). Overexpression of GSK3β in transgenic mice revealed a reduced volume of the entire brain, consistent with the previous findings that the brain volumes of schizophrenia patients were smaller than the healthy controls (Spittaels et al., 2002; Steen et al., 2006). Collectively, these data have suggested that the abnormally high activity of GSK3β is a possible risk factor for schizophrenia susceptibility.

We also observed another risk SNP (rs13320980, MAF = 0.0557) in our samples, and this SNP is in very low linkage with rs3755557 ($r^2 \leq 0.01$, Fig. 2), suggesting that the association of rs13320980 is independent from that of rs3755557. Since the prediction analysis did not reveal any potential function for this SNP, whether it plays any functional role for GSK3β is yet to be dissected.

The observed weak association of the risk SNPs (rs3755557 and rs13320980) in this study argued that either the common variants in GSK3β gene contribute only a small portion to schizophrenia susceptibility.
susceptibility or there are nearby causal SNPs which are responsible for disease susceptibility. We also tested another functional SNP (rs334558) close to rs3755557 [Kwock et al., 2005], but observed no association in our samples, suggesting that the involvement of GSK3β in the etiology of schizophrenia is more complicated than expected.

The imbalance of neurogenesis in the early stage of development of central nervous system has been suggested as a cause of schizophrenia, in which abnormalities of Wnt signaling and PI3K/Akt signaling in schizophrenia have been reported [Cotter et al., 1998; Emamian et al., 2004]. In recent years, the key components in these pathways, such as Akt1, Fzd3, Dkk4 and Apc etc have been found implicated in schizophrenia [Yang et al., 2003; Cui et al., 2005; Xu et al., 2007; Proitsi et al., 2008]. Hence, the likelihood of GSK3β’s involvement in schizophrenia is high though we have only detected a weak association in our samples. It should be noted that the data presented is limited, and we are cautious in the interpretation of our results. Although we have tested two independent samples (more than 2000 individuals) in our study, the sample size is still relatively small compared to the current large-scale genetic studies [Li et al., 2011]. On the other hand, considering that schizophrenia is a disorder almost exclusively originated from brain functions, the functional data from the Hek293T and HeLa cells need to be tested in the neuronal cells. In addition, the observed association of rs3755557 with schizophrenia is weak although it is a functional SNP influencing the expression of GSK3β. Further studies are needed to delineate the role of GSK3β in schizophrenia.

Role of funding source

This work was supported by grants from the National 973 project of China (grant numbers, 2011CB040041 and 2007CB110570), the National Natural Science Foundation of China (grant numbers, 30871343 and 30701101), and the National Science Foundation of Yunnan Province of China (grant number, Y003721111).

Contributors

Authors ML, XJL and BS designed the experiments. Authors ML, YM, XX, XJL, LS and YM performed the experiments. Authors ML and XX analyzed the data. Authors ML, BS, XX and XJL wrote the paper. Authors YM, XYL, LDY and HBD conducted the sample collection. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgements

We are grateful to the volunteers who participated in the genetic studies summarized in this article. We thank the participating psychiatrists in the Second People’s Hospital of Yunnan University for helping with the ascertainment of the study subjects (Dr. Shun-ying Yang, Xing-fu Pu and Jin Yu). We also thank Hui Zhang from Kunming Institute of Zoology for her technical assistance.

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Supplementary data to this article can be found online at doi:10.1016/j.schres.2011.09.013.

