Functional Variants in the Promoter Region of Chitinase 3–Like 1 (CHI3L1) and Susceptibility to Schizophrenia

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The chitinase 3–like 1 gene (CHI3L1) is abnormally expressed in the hippocampus of subjects with schizophrenia and may be involved in the cellular response to various environmental events that are reported to increase the risk of schizophrenia. Here, we provide evidence that the functional variants at the CHI3L1 locus influence the genetic risk of schizophrenia. First, using case-control and transmission/disequilibrium–test (TDT) methodologies, we detected a significant association between schizophrenia and haplotypes within the promoter region of CHI3L1 in two independent cohorts of Chinese individuals. Second, the at-risk CCC haplotype (P = .00058 and .0018 in case-control and TDT studies, respectively) revealed lower transcriptional activity (P = 2.2 × 10−7) compared with neutral and protective haplotypes. Third, we found that an allele of SNP4 (rs4950928), the tagging SNP of CCC, impaired the MYC/MAX-regulated transcriptional activation of CHI3L1 by altering the transcriptional-factor consensus sequences, and this may be responsible for the decreased expression of the CCC haplotype. In contrast, the protective TTG haplotype was associated with a high level of CHI3L1 expression. Our findings identify CHI3L1 as a potential schizophrenia-susceptibility gene and suggest that the genes involved in the biological response to adverse environmental conditions are likely to play roles in the predisposition to schizophrenia.

Schizophrenia (MIM 181500) is a common, complex major mental disorder affecting ∼1% of the population worldwide. Clinical and epidemiological investigations indicate that schizophrenia is a multifactorial disorder with genetic and environmental elements contributing to overall risk. A number of environmental factors, including obstetric complications, prenatal exposure to infection or famine, and psychosocial stressors, have been reported as risk factors of schizophrenia. An adverse environment can produce physiological and/or psychological stress, which, in turn, may result in brain dysfunctions. A key period of vulnerability to environmental stress may be during brain development. This is consistent with current concepts that view schizophrenia as primarily a disorder of neurodevelopment. However, sensitivity to environmental stressors shows substantial interindividual variation, and at least part of this variation may be genetic in origin and/or involve gene-environment interactions.

The chitinase 3–like 1 gene (CHI3L1 [MIM 601525]), located on chromosome 1q32.1, a region that shows weak linkage to schizophrenia, has been reported to display disease-specific alteration in gene expression in the hippocampus of patients with schizophrenia. One function of CHI3L1 is to act as a cellular survival factor in response to an adverse environment: various types of physiological stress, such as inflammation, hypoxia, and nutrient deprivation, may induce high expression of CHI3L1. The presence of CHI3L1 protein inhibits the activity of ASK1, which is pivotal for the transmission of stress-induced cellular responses, and protects the cells from undergoing apoptosis.

Here, we present evidence that CHI3L1 contributes to the genetic risk factors of schizophrenia. We have observed significant association between schizophrenia and genetic variations in the CHI3L1 locus in both family-based and population-based investigations. Moreover, the CHI3L1 haplotypes implicated in schizophrenia susceptibility were associated with the altered expression level of the gene. Cis-acting effects of the functional variants in CHI3L1 may be responsible for the association.

Material and Methods

Subjects

For the case-control investigation, 412 unrelated patients with schizophrenia and 464 control individuals were recruited in this study. The patient group consisted of 200 men and 212 women with a mean ± SD age of 41.13 ± 12.97 years. The control group consisted of 200 men and 264 women with a mean age of 34.88

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± 10.37 years. A subgroup of samples including 29 patients (15 men and 14 women with a mean age of 39.68 ± 12.66 years) and 54 controls (27 men and 27 women with a mean age of 39.66 ± 11.99 years) was used to investigate gene expression. All the population-based samples were from Shanghai, East China. For the transmission/disequilibrium–test (TDT) study, 308 unrelated probands with schizophrenia and their biological parents were recruited. The probands consisted of 163 males and 145 females with a mean age of 24.0 ± 6.6 years, of whom 128 were from Shanghai, 65 from Xi’an, 35 from Changchun, and 80 from Jilin. Subjects with schizophrenia were given strict diagnoses according to the criteria of the DSM-IV, with the use of a combination of examination of psychiatric case records and clinical interviews, which used the Chinese version of the Schedule for Affective Disorders and Schizophrenia–Lifetime (SADS-L). The diagnoses were checked and verified by another independent senior psychi- atrist who reviewed the psychiatric case records. A total of 1,800 subjects, including 720 patients with schizophrenia, were recruited in this study. All subjects were Han Chinese in origin. Participants gave a standard informed consent in the protocol, which was reviewed and approved by the Shanghai Ethical Com- mittee of Human Genetic Resources, after the nature of the study had been fully explained.

Genotyping

All five SNPs were selected from dhSNP. SNP1, SNP2, and SNP5 were scored using a single-nucleotide primer extension (SNuPe) method with MegaBACE SNuPe genotyping kit (Amersham Biosciences) and the primers that anneal 1 b upstream of the target SNPs. A complementary-labeled deoxyinosine triphosphate was added at the SNP site in the reaction. The SNuPe products were then cleaned and were electrophoresed on MegaBACE 1000 instruments (Amersham Biosciences). SNP3 and SNP4 were genotyped using direct sequencing on ABI 3100 instruments (Applied Biosystems).

Cell Culture, Transfection, and MYC-Inhibitor Trial

U251 cells were cultured in Dulbecco’s modified Eagle medium (Gibco-BRL) with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C and 5% CO₂. Transient transfection for the luciferase reporter assay was performed in a 48-well plate with use of LipofectAmine 2000 regent (Invitrogen), and pCBR control luciferase reporter assay was performed in a 48-well plate with use of T4 polynucleotide kinase. The binding reaction contained 8 μg of nuclear-extract proteins, 1 x binding buffer, and, if needed, unlabeled competitors; labeled probes were added after 10 min, and samples were incubated at room temperature for a total of 30 min. For the supershift assay, nuclear-extract proteins, labeled probes, and antisera were incubated for 16 h at 4°C. Samples were analyzed by electrophoresis on 4% (w/v) native polyacrylamide gels. The gels were dried and were autoradiographed for 3 h or overnight.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using Trizol reagent. RNA integrity was confirmed by direct visualization of 18S and 28S rRNA bands after agarose-gel electrophoresis. RNA samples were incubated using 10 units of DNase I (Novagen) at 37°C for 20 min to remove residual DNA, followed by inactivation at 65°C for 10 min. RNA samples were further purified using a HiBind spin column (Omega) in accordance with the manufacturer’s instructions. The purified RNA samples (0.5 μg) were then reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen) and random hexamers.

Real-Time Quantitative PCR and Allele-Specific Expression Assay

Real-time PCR was performed using the ABI7900 system (Applied Biosystems). Reactions were performed in a 10-μl volume including diluted cDNA samples, primers, and SYBR Green I Mastermix (Applied Biosystems). Diluted cDNA samples produced from 10 ng total RNA were added to each well. Real-time PCR data were collected using SDS software (version 2.1 [Applied Biosystems]). Expression of CHI3L1 was normalized in relation to GAPDH levels (denoted as ΔΔCt). Both GAPDH and CHI3L1 were tested four times for each sample. SNP5 within exon 5 was used as a marker for the allele-specific expression assay. The SNuPe method was used to compare relative allelic expression in individual subjects who were heterozygous for the marker polymorphisms. To normalize the signal from different fluorescent dyes, genomic DNA with a perfect 1:1 ratio of the two alleles was used to correct allelic ratios obtained from each cDNA analysis. The SNuPe products of DNA and cDNA templates from the same samples were electrophoresed in the same capillaries in one run, by multiple injections.

Risperidone Trial

Risperidone (Xian Janssen-Cliag) was dissolved in saline and was administered to C57Bl/6 mice (1 mg/kg) daily. Control mice received the vehicle alone. All the mice were housed, five per cage, at room temperature with a 12-h light/dark cycle. For each group, 5 male and 5 female mice were included. Mice received risperi- done or the vehicle for 3 wk and were killed 2 h after the final trial.

Figure 1. Genomic structure of CHI3L1 and the location of SNPs included in the present study.
Table 1. Genetic Association Analyses of the CHI3L1 Gene with Schizophrenia

<table>
<thead>
<tr>
<th>Allele/Haplotype</th>
<th>Marker and Alleles or Haplotype</th>
<th>dbSNP Identification</th>
<th>Case-Control Study No. and Allelic Frequency (%)</th>
<th>Odds Ratio</th>
<th>TDT Study a</th>
<th>P b</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>SNP1 (C/T)</td>
<td>rs2364574</td>
<td>Patients: 224 (28.4) Controls: 237 (25.8)</td>
<td>1.14</td>
<td>.24</td>
<td>...</td>
</tr>
<tr>
<td>T</td>
<td>SNP2 (C/T)</td>
<td>rs6691378</td>
<td>Patients: 234 (29.4) Controls: 316 (34.3)</td>
<td>.80</td>
<td>.028</td>
<td>178/208 (.86)</td>
</tr>
<tr>
<td>T</td>
<td>SNP3 (C/T)</td>
<td>rs10399605</td>
<td>Patients: 227 (28.7) Controls: 313 (34.1)</td>
<td>.78</td>
<td>.016</td>
<td>161/198 (.81)</td>
</tr>
<tr>
<td>C</td>
<td>SNP4 (G/C)</td>
<td>rs495908</td>
<td>Patients: 137 (17.4) Controls: 114 (12.4)</td>
<td>1.49</td>
<td>.0039</td>
<td>88/56 (1.57)</td>
</tr>
<tr>
<td>G</td>
<td>SNP5 (G/A)</td>
<td>rs880632</td>
<td>Patients: 303 (37.2) Controls: 340 (36.9)</td>
<td>1.01</td>
<td>.88</td>
<td>...</td>
</tr>
<tr>
<td>CCG</td>
<td>SNP2 haplotype</td>
<td>...</td>
<td>Patients: 398 (50.7) Controls: 475 (51.8)</td>
<td>.97</td>
<td>.67</td>
<td>315/310 (1.01)</td>
</tr>
<tr>
<td>TTG</td>
<td>SNP3 haplotype</td>
<td>...</td>
<td>Patients: 204 (26.1) Controls: 289 (31.5)</td>
<td>.77</td>
<td>.013</td>
<td>153/187 (1.82)</td>
</tr>
<tr>
<td>CCC</td>
<td>SNP4 haplotype</td>
<td>...</td>
<td>Patients: 135 (17.2) Controls: 104 (11.3)</td>
<td>1.62</td>
<td>.00058</td>
<td>93/57 (1.63)</td>
</tr>
<tr>
<td>Global</td>
<td></td>
<td>...</td>
<td>Patients: ... Controls: ...</td>
<td>...</td>
<td>.0018</td>
<td>...</td>
</tr>
</tbody>
</table>

a P values, not corrected for multiple tests, in population-based samples.
b Number of transmitted (T) and untransmitted (NT) alleles or haplotypes. T/NT ratios are shown in parentheses.

Table 2. Estimates of LD Statistics between All Pairs of Markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>SNP1</th>
<th>SNP2</th>
<th>SNP3</th>
<th>SNP4</th>
<th>SNP5</th>
</tr>
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<tr>
<td>SNP1</td>
<td>.89</td>
<td>.81</td>
<td>.29</td>
<td>.61</td>
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<tr>
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<td>.94</td>
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<td>.07</td>
<td>.07</td>
<td>.04</td>
</tr>
<tr>
<td>SNP5</td>
<td>.08</td>
<td>.05</td>
<td>.04</td>
<td>.10</td>
<td></td>
</tr>
</tbody>
</table>

a D' values are shown above the diagonal; r² values are shown below the diagonal. LD values in family trios are shown after the slash.

Figure 2. Luciferase reporter assay of SNP2, SNP3, and SNP4 common haplotypes in U251 cells. The transcriptional activity of the pCBG99 control was set at 100%. Each CHI3L1 haplotype was tested eight times. Among the haplotypes, the risk haplotype (CCC) revealed the lowest activity, whereas the protective haplotype (TTG) showed the highest activity (P = 2.2 × 10⁻⁷, by one-way ANOVA).
SNP3 and SNP4 are located in the core promoter region of CHI3L1. We used EMSAs to determine whether the genetic variants would alter DNA-protein interaction. The probes of both alleles of SNP3 produced one binding complex with U251 nuclear-extract proteins, but there was no difference between the two alleles (fig. 3a). As for SNP4, the C-allele probe generated one clear binding complex with nuclear extract, but the G-allele probe generated two. The additional complex with lower mobility can still remain visible after the 100× C-allele cross-competitor is loaded (fig. 3b).

The SNP4 G→C transversion appeared to alter the consensus sequences of several transcription factors, on the basis of analyses with Genomatix software. We predicted that MYC/MAX and AP-2 would be promising candidates and performed supershift analysis with use of the monoclonal antibodies to MYC and AP-2. The addition of either of these antibodies resulted in weakness of the G-allele-specific band; however, the intensity of the supershifted bands was too low for reliable visualization. Lengthening of the exposure time (overnight) enabled good observation of the supershift bands (fig. 2c). AP-2 is a negative regulator to MYC-mediated transcriptional activation when their binding sites are adjacent. Hence, our further investigation was focused on the role of MYC/MAX in CHI3L1 transcription. We treated U251 and THP-1 (human monocytic cell line) cells with MYC inhibitor, which interfered with the interaction between MYC and MAX. We detected a severely decreased CHI3L1 expression in both cell lines (fig. 4), which pointed to an active MYC/MAX binding site in the promoter region of CHI3L1 being involved in controlling the expression of this gene. The SNP4 G→C polymorphism may, therefore, influence gene expression by impairing the MYC/MAX-regulated transcriptional activity. Meanwhile, similar results in the gliar- and lymphoid-derived cells indicated that the effect of SNP4 may be common in the blood and in the brain.

We used real-time PCR to quantify the gene expression...
in the peripheral blood cells (PBC) isolated from 82 individuals. The expression of CHI3L1 was decreased by 19% in 29 patients compared with its expression in 53 controls, but there was no statistically significant difference between the two groups ($P = .081$) (fig. 3a). The samples were then grouped together for analysis. The CHI3L1 expression level was obviously correlated with genotypes of SNP4 ($P = 4.2 \times 10^{-5}$, corrected for 5 tests) (fig. 5b), as well as with genotypes of SNP3 ($P = 7.6 \times 10^{-4}$, corrected for 5 tests) (fig. 5c). We determined the precise haplotypic structures of the 82 samples by cloning and sequencing the DNA fragments containing SNP2, SNP3, and SNP4. There was a significant association between gene expression and common haplotypes ($P = 3.1 \times 10^{-3}$, corrected for 5 tests) (fig. 5d).

To minimize the trans-acting influences, we performed an allele-specific expression assay, using SNP5 as a marker for mRNA transcribed from each chromosomal allele. The objective of the assay was to determine the allele-specific expression pattern of SNP4: the C alleles of SNP4 were almost always in linkage with the A alleles of SNP5 ($D = 0.98$), and, therefore, the comparative expression ratio of A/G in SNP5 represented that of C/G in SNP4 for the samples that were heterozygous in both loci. In total, 33 samples that were heterozygous in SNP5 were used in the current assay. When the data from all the samples were combined, the comparative allelic ratios of A/G were widespread. However, in the six samples that were also heterozygous in SNP4, the expression of the A allele was significantly reduced by $\sim 80\%$ compared with the G allele ($P = 9.1 \times 10^{-6}$, corrected for 5 tests) (fig. 5e). This result further indicated that the C allele of SNP4 was responsible for the reduced CHI3L1 expression. The data from the quantitative PCR and allele-specific expression assay supported the cis-acting role of SNP4 in gene expression in vivo.

Figure 5. Expression analyses of CHI3L1 in vivo. a, Expression level of CHI3L1 in the PBC from patients with schizophrenia and unaffected controls. The average level of CHI3L1 in unaffected controls was defined as 100. No significant difference in expression level between the two groups was detected ($t$ test). b and c, Expression of CHI3L1 was associated with the genotype of both SNP4 (b) and SNP3 (c) in PBC (Kruskal-Wallis $H$ test $P < .01$). d, Haplotypes in the promoter region of CHI3L1 associated with expression level of the gene in PBC. e, Relative allele-specific expression of the SNP5 data expressed as allele ratios of A/G. Patients who were heterozygotes for SNP4 revealed highly significant allele differences ($t$ test, corrected for 5 tests) f, Risperidone-treated mice did not change the mRNA level of CHI3L1 in blood and brain ($t$ test $P > .05$). The expression level of CHI3L1 in untreated mice was defined as 100.
Genetic and environmental risk factors

CHI3L1

AKT signaling

Neural injury
and neurotransmitter
abnormality

Stimulatory
influence

Inhibitory
influence

Predisposition
to schizophrenia

Immune responses
and inflammatory cytokines

Neurodevelopmental
abnormality

Figure 6. Hypothetical scenario for CHI3L1 in the pathophysiology of schizophrenia. Impairment of AKT signaling and over-activation of immune responses were observed in the subjects with schizophrenia and were considered to contribute to the risk of schizophrenia. The CHI3L1 gene may partly compensate the alteration of the processes and, therefore, may play a role in preventing the progression of psychosis.

In view of the possible effects on gene expression data from antipsychotic medication in the probands with schizophrenia, we examined the CHI3L1 expression level after risperidone treatment, since most patients recruited for the expression studies were receiving this treatment. Our results indicated that risperidone treatment does not change the CHI3L1 mRNA level in vivo (fig. 5f).

Discussion

In this study, we observed positive association between schizophrenia and genetic variations in the promoter region of the CHI3L1 gene in two independent cohorts of Chinese patients with schizophrenia. The association remained significant in both population-based and family-based investigations after correction for multiple tests. Our combined findings made it unlikely that they are the result of a type I error and ruled out the possibility that stratification could explain the results. However, we cannot formally exclude the possibility that the observed association signal originates from genes in the vicinity of the CHI3L1 locus and not from the CHI3L1 locus itself. Further detailed LD-mapping studies performed in different populations are essential. Nevertheless, CHI3L1 is a promising candidate gene for schizophrenia, and the parallel functional evidence reinforces our positive association findings. We have demonstrated that the individuals carrying risk alleles/haplotypes have lower expression levels of CHI3L1 than do those carrying protective alleles/haplotypes. The effect is also greater in individuals carrying two copies of the risk alleles/haplotypes. Our results strongly indicate that susceptibility variants in the CHI3L1 locus are likely to exert their effect mainly by changing the abundance of gene production. Furthermore, SNP4, which gives the strongest association with schizophrenia, is a good candidate for such a susceptibility variant, since the G–C transversion impairs the MYC/MAX–regulated transcriptional activity, which, in turn, can account for the reduced expression of the risk haplotype.

The genetic variations that change the expression of CHI3L1 may influence some key processes that are CHI3L1-dosage dependent. One is activation of AKT-mediated signal pathways through phosphatidylinositol-3 kinase (PI-3K)–dependent phosphorylation.18 There is strong convergent evidence indicating that the PI-3K/AKT signal pathway is implicated in schizophrenia; AKT1-GSK3β, a component of AKT-mediated signals pathway, is impaired in patients with schizophrenia, and antipsychotic medication may induce AKT activation, to compensate for the impairment.19 An additional possibility is that CHI3L1 participates in the pathophysiology of schizophrenia through its function on the immune response. Activation of the inflammatory response system has been observed in schizophrenia and affective disorders.20,21 High levels of inflammatory cytokines, the immune mediators, have an effect on neuronal survival22 and result in neurobehavioral deficits in animal models.23 CHI3L1 may have an inhibitory effect on these inflammatory responses24 and provides a negative-feedback control for the action of inflammatory cytokines.10 These functional observations suggest a role of the CHI3L1 gene in preventing the progression of psychosis (fig. 6), whereas the functional variations that reduce expression levels may increase the genetic risk of developing schizophrenia.

AKT and cytokines, as well as CHI3L1, play a role in cell response to an adverse environment; it has long been known that the production of inflammatory cytokines is stimulated by the stress coupled with adverse environmental events.21 The AKT pathway has been strongly associated with cell survival25,26 and regulates the cytokine-induced cellular responses.27 Certain genes involved in PI-3K/AKT and cytokine signaling have been reported to be associated with schizophrenia.19,28–30 It will be of interest to investigate the interaction between these genes and CHI3L1 under adverse environmental circumstances and the possible effects of epistasis between the genes on the genetic susceptibility to schizophrenia.

In conclusion, our findings identify CHI3L1 as a potential schizophrenia-susceptibility gene. Data from genetic and molecular analyses provide clear and consistent evidence for the functional SNP4 to be a strong candidate for a risk factor of schizophrenia. Moreover, our results, together with the previously published observations, support the proposal that genes involved in biological response to adverse environmental conditions play roles in the predisposition to schizophrenia.
Acknowledgments
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Web Resources
The URLs for data presented herein are as follows:
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for schizophrenia and CHI3L1)

References