Evidence for association between the 5′ flank of the NOS1 gene and schizophrenia in the Chinese population

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Abstract

Nitric oxide (NO) plays an important role in the dopaminergic and serotonergic system as the second messenger of the NMDA receptor and has possible roles in neurotransmission, neurosecretion, synaptic plasticity, and tissue injury in many neurological disorders, including schizophrenia. There is also genetic evidence to support the human NOS1 (neuronal nitric oxide synthase 1) gene as a promising candidate gene associated with schizophrenia. In this paper we conducted a case-control association study involving 1705 Chinese subjects and 12 genetic markers [11 single nucleotide polymorphisms (SNPs) and 1 microsatellite] mainly in the 5′ flank region of the gene by direct sequencing and capillary electrophoresis. We identified SNP rs3782206 and several haplotypes derived from it as being significantly associated with schizophrenia and, specifically, in a paranoid subgroup. Our results strongly support a previous hypothesis that NOS1 contributes to the genetic risk of schizophrenia and suggest that further research on more NOS1 variants and its regulatory elements are warranted.

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Key words: Association, case-control studies, Chinese population, nitric oxide, NOS1, schizophrenia.

Introduction

Schizophrenia (MIM 181500) is a common and devastating psychiatric disorder that affects approximately 1% of the population worldwide (Saha et al., 2005). Symptoms of the disease include delusion, hallucination accompanied in varying degrees by other emotional, behavioural, or intellectual disturbances. Schizophrenia, as well as being affected by environmental factors is a mental disorder which is highly heritable. Linkage and association studies have been widely used to identify the relevant genes and susceptibility region.

There is an increasing body of evidence that the biological messenger, nitric oxide (NO) is involved in the physiological and pathological function of the central nervous system (Brenman et al., 1997). NO is the second messenger of the N-methyl-D-aspartate (NMDA) receptor, and 90% of NO is catalysed by NOS1 (neuronal nitric oxide synthase 1) (Brenman et al., 1997; Garthwaite et al., 1989). Evidence suggests that the NO pathway is connected to the dopamine system in the pathogenesis of schizophrenia (Lorrain and Hull, 1993). In a recent study, Yao and colleagues identified a significant increase in NO in brain samples of schizophrenia patients compared with normal subjects, a result which was confirmed in serum tests (Herken et al., 2001; Yao et al., 2004; Yilmaz et al., 2007). Pharmacological studies in animal models, point to the nitrinergic system as the means whereby NO and its pathway are involved both in schizophrenic and schizoaffective disorders not only...
through dopamine but also through the serotonin system (Bird et al., 2001; Black et al., 1999; Bujas-Bobanovic et al., 2000a, b; Klamer et al., 2004). Lauer et al. (2005) found that nitrinergic neurons are pathologically altered in schizophrenia patients, a finding confirmed by Fritzen et al. (2007) using even larger samples.

12q22-24 is a hot linkage location of psychotic disorder. Studies have identified nine instances in bipolar disorder samples, one in major depression and three in schizophrenia and schizoaffective disorder (Abkevich et al., 2003; Baier et al., 2000, 2002; Brzustowicz et al., 2000; Cassidy et al., 2007; Curtis et al., 2003; Degen et al., 2001; DeLisi et al., 2002; Detera-Wadleigh, 1999; Ewald et al., 1998, 2002; Maziade et al., 2005; Morissette et al., 1999; Shink et al., 2005). A number of association studies have identified NOS1 as a candidate gene of schizophrenia but others have not supported these findings (Fallin et al., 2005; Liou et al., 2003; Reif et al., 2006; Shinkai et al., 2002). The human NOS1 gene has a complex structure which is characterized by its 12-alternative untranslated exon 1, termed exon 1a-1l. Expression of the different exon1-related splice variants of NOS1 mRNA is controlled directly by the associated 5’ flanking sequences (Bros et al., 2006). A variable number tandem repeat (VNTR) in exon 1f within the promoter region of NOS1 has been suggested as being functional and affected the severity of the disease, and an exon 1c promoter SNP (G-84A) was associated with schizophrenia (Reif et al., 2006). A promoter region >130 kb, NOS1 has a coding area containing a further 110 kb composed of 28 exons. A synonymous single nucleotide polymorphism (SNP) (rs2682826) in exon 29, a C->T transition located 276 bp downstream from the translation termination site of the NOS1 gene, has been extensively investigated and has been shown to be strongly significant (p =0.000007) with schizophrenia in a study of 215 Japanese patients and 182 health controls (Buttenschon et al., 2004; Liou et al., 2003; Shinkai et al., 2002; Yu et al., 2003). Recently Fallin et al. (2005) selected nine SNPs spanning NOS1 and identified a 4-SNP haplotype as being potentially associated with schizophrenia and schizoaffective disorder (p<0.001). However, there has so far been no evidence of association with bipolar disorder and major depression (Bruzstowicz et al., 2000; Buttenschon et al., 2004; Yu et al., 2003).

We conducted a systematic case-control association study to investigate mainly the 5’ flank region of the NOS1 gene using larger sample sizes than before and more genetic markers in the Chinese population.

Material and methods

Subjects

For the initial association study we recruited 480 schizophrenia patients (262 males, 218 females, mean age 46±12 yr) and 480 controls (254 males, 226 females, mean age 35±8 yr). Following some interesting initial results, we increased the sample size to 844 patients (447 males, 397 females, mean age 46±13 yr), containing 425 paranoid-type patients (222 males, 205 females, mean age 47±22 yr; S-P group) and 861 health controls (466 males, 395 females, mean age 44±9 yr). All subjects were of Chinese Han origin. A clinical interview was administered to all subjects including cases and controls by a senior psychiatrist of the Shanghai Institute of Mental Health. A final diagnosis was made by two independent psychiatrists from the Shanghai Institute of Mental Health on the basis of data from the interview and hospital case-notes. All patients met DSM-IV criteria for schizophrenia and without substance use and organic mental syndromes. According to the criteria, we specified five subtypes of schizophrenia: paranoid (S-P), hebephrenic (S-H), catatonic (S-C), undifferentiated (S-U), and residual (S-R). After the procedure had been fully explained, written informed consent was obtained from all participants in the study which was reviewed and approved by the Shanghai Ethics Committee of Human Genetic Resources.

Variants selection and genotyping

The human NOS1 gene spans 150 kb with 29 exons. We selected nine SNPs and one VNTR spanning ~130 kb in the 5’ flank region of the NOS1 gene containing the 5’ upstream region, the whole promoter region and the first three exons. All the SNPs were selected by Tag SNP Picker (http://www.hapmap.org/) with r2 >0.8 and minor allele frequency cut-off was 0.08. Four SNPs used to construct the haplotype in the Fallin et al. (2005) study were also included in our study. In addition, we also selected another two SNPs in exon 29 since several previous studies had investigated the two synonymous SNPs. In total, 12 genetic markers were chosen in the genomic structure of NOS1. Figure 1 shows the genetic information of the 12 markers.

Genomic DNA was extracted from peripheral blood using a modified phenol/chloroform method. Polymerase chain reactions (PCRs) were carried out in 96-well microtitre plates with a final 25 µl reaction volume containing 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 1.5 mM MgCl2, 200 mM dNTPs, 5 µl Q
solution (Qiagen, Valencia, CA, USA), 10 pm of each primer, 20 ng DNA, and 2.5 U Taq polymerase (Life Technologies, Karlsruhe, Germany). PCR conditions consisted of an initial 5 min at 95 °C, 35 cycles of 94 °C for 30 s, annealing temperature 57 °C for 40 s, 72 °C for 50 s, and a final extension period of 10 min at 72 °C using the GeneAmp PCR System 9700 (Applied BioSystems, Foster City, CA, USA). All the SNPs were genotyped using DNA sequencing on an ABI 3100 genetic analyser using the ABI Prism BigDye Terminator Cycle Sequencing kit version 3.1 (Applied BioSystems). PCR amplification of microsatellite markers VNTR in exon-1f was performed using primers fluorescently labelled with FAM. PCR products were electrophorized on MegaBACE 1000 instruments and analysed using Genetic profiler software (Amersham Biosciences, Piscataway, NJ, USA). Alleles were dichotomized into short (S) and long (L) alleles as described by Reif et al. (2006) with up to nine repeats (i.e. the I allele) being designated as short alleles given the different allelic distribution between the different ethnic populations (data not shown).

Statistical analyses

The difference in distribution of allele and genotype frequencies between cases and controls and Hardy–Weinberg equilibrium (HWE) were computed on SHEsis (http://analysis.bio-x.cn). The presence of HWE was determined using the $\chi^2$ test for goodness of fit. Linkage disequilibrium (LD) between polymorphisms and haplotype block structures was evaluated on Haploview software version 3.11 (http://www.broad.mit.edu/mpg/haploview/index.php). The haplotype analysis was performed using the COCAPHASE command implemented on UNPHASED program version 2.43 (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/). Expected maximum (EM), estimate missing genotypes and droprare options were used. To deal with multiple testing, allelic associations were evaluated by permutation test (10000 permutations) implemented in Haploview. All tests were two-tailed and significance was accepted at $p < 0.05$. Power calculations were performed using the G*Power program.

Results

No deviation of HWE was found in the genotypic distribution of any polymorphism, except for rs2682826, which showed a significant deviation ($p = 0.008$) in the control group. Two SNPs, rs499776 and rs3782206, showed allelic association with schizophrenia (nominal $p = 0.014$, $p = 0.015$, respectively) and rs561712 showed a trend towards association ($p = 0.054$) in the analysis with 480 cases and 480 controls (Table 1). These three SNPs covered 35 kb of the NOS1 5' flank region, and rs3837437 was also within this region. We therefore genotyped the four interesting SNPs, rs499776, rs3782206, rs3837437 and rs561712 in a total sample of 1705 (861 controls and 844 cases). We identified a more significant association of rs3782206 with schizophrenia (nominal $p = 0.004$, $p = 0.014$ after permutation). In the S-P subgroup, we

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**Figure 1.** Genomic structure and linkage disequilibrium (LD) between markers genotyped in NOS1 and their position. LD structure ($\text{LD}^\prime$) between marker pairs is indicated by the shaded matrices.
also found significant deviation for rs3782206 (nominal \( p = 0.012 \), \( p = 0.048 \) after permutation) (Table 2).

We constructed haplotypes of rs499776, rs3782206, rs3837437 and rs561712 and then carried out two-, three- and four-marker tests of haplotypic association in the sample of 1705 subjects and the S-P subgroup, and found a number of significant results. The 4-SNP haplotype reached a nominally significant global \( p \) value of 0.0028, and two risk individual haplotypes G-G-T-C and G-G-T-T obtained a nominally \( p \) value of 0.0058 and 0.0069, respectively. The core haplotype composed of rs3837437 and rs3782206 with T-C presented the strongest significance in both samples (844 cases and 861 controls) (nominal \( p = 0.0002 \), \( p = 0.0026 \) after permutation) and S-P subgroup (nominal \( p = 0.0006 \), \( p = 0.0017 \) after permutation), which
Table 2. Allelic and genotypic test in the 1st initial, 2nd confirmatory, total, and S-P subgroup sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>rs499776</th>
<th>rs561712</th>
<th>rs3837437</th>
<th>rs3782206</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Allele (%)</td>
<td>OR</td>
<td>Allele (%)</td>
<td>OR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>p</td>
<td>A</td>
</tr>
<tr>
<td>1st</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>480</td>
<td>162 (0.17)</td>
<td>784 (0.83)</td>
<td>0.75</td>
<td>201 (0.21)</td>
</tr>
<tr>
<td>Control</td>
<td>480</td>
<td>204 (0.22)</td>
<td>742 (0.78)</td>
<td>0.015</td>
<td>233 (0.25)</td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>364</td>
<td>141 (0.21)</td>
<td>547 (0.79)</td>
<td>1.05</td>
<td>153 (0.23)</td>
</tr>
<tr>
<td>Control</td>
<td>381</td>
<td>147 (0.20)</td>
<td>599 (0.80)</td>
<td>0.709</td>
<td>144 (0.20)</td>
</tr>
<tr>
<td>Total</td>
<td>844</td>
<td>303 (0.18)</td>
<td>1331 (0.82)</td>
<td>0.87</td>
<td>354 (0.22)</td>
</tr>
<tr>
<td>Control</td>
<td>861</td>
<td>351 (0.21)</td>
<td>1341 (0.79)</td>
<td>0.11</td>
<td>377 (0.23)</td>
</tr>
<tr>
<td>S-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>425</td>
<td>160 (0.19)</td>
<td>662 (0.81)</td>
<td>0.92</td>
<td>175 (0.21)</td>
</tr>
<tr>
<td>Control</td>
<td>861</td>
<td>351 (0.21)</td>
<td>1341 (0.79)</td>
<td>0.454</td>
<td>377 (0.23)</td>
</tr>
</tbody>
</table>

SNP, Single nucleotide polymorphism; OR, odds ratio.

* p = 0.014 after 10000 permutations.

b p = 0.048 after 10000 permutations.

Further support for NOS1 gene in schizophrenia.
implied that T-C may be a protective haplotype (cases 53.6% vs. controls 59.7%). Details of haplotypic association result are shown in Table 3.

Discussion

In spite of the many association studies in the field of schizophrenia, it is still unclear which genes are really involved and how they interact with each other. This is partly due to the problems involved in replicating studies as well as to the complicated nosogenesis of the disease. In our current study, we attempted to confirm earlier research implicating \textit{NOS1} at 12q24 as a susceptibility gene for schizophrenia. We selected 10 genetic variants in the 5' flank region of \textit{NOS1} and two variants in its 29th exon, and identified a significant association of \textit{NOS1} with the disease.

We found significant differences between patients and controls in the allele frequency of rs3782206 in our total sample of 1705 subjects ($p = 0.014$ after permutation). Our sample size was sufficient to detect an odds ratio of 1.25 with 65% power or 1.5 with 88% power. The strongest haplotypic associations found were in haplotypes of rs3837437 and rs3782206 ($p = 0.0002$ after permutation), which implied that association might still be present with a nearby locus because of LD. Although rs3782206 did not show a positive result in the Fallin et al. (2005) study of an Ashkenazi Jewish population, both studies found that an overlapping haplotype region composed of rs561712 and rs3782206 was positive in schizophrenia samples (Figure 1). The ethnic groups in the two studies shared a similar LD structure in the NOS1 region. This would indicate that NOS1 was associated with schizophrenia and, in particular, the 5' flank region with promoter function of this gene could play a vital role in the pathology and aetiology of schizophrenia.

We failed to replicate the result of Shinkai et al. (2002) in rs2682826 but this might simply reflect the problems of replication inherent in association studies of complex diseases. Such differences might be due to a range of problems, such as ethnic stratification, difference of phenotype definition and most importantly, sample size. Our sample size was much bigger than Shinkai et al.’s (215 patients and 186 controls), and our results should therefore be more robust.

The promoter region of NOS1 is the main regulating element involved in the pathogenesis of schizophrenia.

### Table 3. Haplotype analysis of four single nucleotide polymorphisms in the total sample (844 cases and 861 controls) and S-P subgroup sample

<table>
<thead>
<tr>
<th>No. of markers</th>
<th>Global haplotype reference and sample</th>
<th>Estimated haplotype frequency (%)</th>
<th>Individual haplotype $p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3837437–rs3782206</td>
<td>rs3837437–rs3782206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sample</td>
<td>0.0012</td>
<td>T/C</td>
<td>53.6</td>
</tr>
<tr>
<td>S-P subgroup</td>
<td>0.0035</td>
<td>T/C</td>
<td>52.7</td>
</tr>
<tr>
<td>rs561712–rs3837437–rs3782206</td>
<td>rs561712–rs3837437–rs3782206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sample</td>
<td>0.0015</td>
<td>G/T/C</td>
<td>32.0</td>
</tr>
<tr>
<td>S-P subgroup</td>
<td>0.0058</td>
<td>G/T/C</td>
<td>31.3</td>
</tr>
<tr>
<td>rs499776–rs561712–rs3837437–rs3782206</td>
<td>rs499776–rs561712–rs3837437–rs3782206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sample</td>
<td>0.0028</td>
<td>G/G/-/-C</td>
<td>17.4</td>
</tr>
<tr>
<td>S-P subgroup</td>
<td>0.0048</td>
<td>G/G/-/-C</td>
<td>18.5</td>
</tr>
</tbody>
</table>

* $p$ value by 10000 permutations test.
schizophrenia. Using a Go/No-go paradigm of electrophysiology and neuropsychological testing of severely affected patients, Reif et al. (2006) observed that the promoter polymorphisms had an impact on prefrontal functioning. Our own haplotype association analysis also supported this hypothesis since we found that rs499776–rs561712–rs3837437–rs3782206 covering the promoter region and the first three exons showed a strong positive association.

As a hot linkage spot for schizophrenia and bipolar disorder, the linkage signal of 12q24 is rather broad and has no prominent single peak (Reif et al., 2006). This implies that several risk genes might locate in this region, with each gene having a relatively modest influence. Another promising gene in this region might be DAAO. A previous study by our group found that this gene had a significant association with schizophrenia (Liu et al., 2004). It could be interesting to test whether these two genes were synergistically interacting or acting independently.

Moreover, CAPON (NOS1AP, neuronal nitric oxide synthase 1 adaptor protein) is an adaptor protein, regulating the coupling of NOS1 to the NMDA receptor through the PSD95 domain whose expression in turn is decreased in schizophrenia and bipolar disorder subjects compared to normal controls (Clanton and Meador-Woodruff, 2004). It has been identified as a putative candidate gene for schizophrenia in studies of three independent ethnic groups (Brzustowicz et al., 2004; Miranda et al., 2006; Zheng et al., 2005). In addition, a number of reports have identified NMDA receptor subunits as being associated with schizophrenia. It may be that epistatic interaction between NOS1 and CAPON influences the regulation of the glutamate system.

In summary, we identified the 5' flank region of NOS1 and related haplotypes as being associated with schizophrenia. Further work might focus on the functional role of NOS1 in the pathogenesis of schizophrenia.

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Statement of Interest

None.

References


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