

ORIGINAL RESEARCH ARTICLE

Allelic association of the neuronal nitric oxide synthase (NOS1) gene with schizophrenia

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Nitric oxide (NO) has been identified as a widespread and multifunctional biological messenger molecule in the central nervous system (CNS), with possible roles in neurotransmission, neurosecretion, synaptic plasticity, and tissue injury in many neurological disorders, including schizophrenia. Neuronal NO is widely produced in the brain from L-arginine catalyzed by neuronal NO synthase (NOS1). We therefore hypothesized that the NOS1 gene may play a role in the pathophysiology of schizophrenia. In the present study, we examined the genetic association between a novel single nucleotide polymorphism (SNP: a C→T transition located 276 base pairs downstream from the translation termination site) of the human NOS1 gene, which is located in chromosome 12q24, and schizophrenia (215 Japanese patients with schizophrenia and 182 healthy controls). The allele frequencies of the polymorphism in exon 29 of the NOS1 gene differed significantly between patients with schizophrenia and controls ($\chi^2 = 20.10$, $df = 1$, $P = 0.000007$; relative risk = 1.92; 95% confidence interval = 1.44–2.55). Our results suggest that the NOS1 gene polymorphism may confer increased susceptibility to schizophrenia.

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Nitric oxide (NO) is a gaseous, widespread, and multifunctional biological messenger molecule, and is one of the oxyradicals (for reviews, see Krukoff¹ and Yun *et al.*²). NO has been accepted as a nonconventional neurotransmitter in the central nervous system (CNS), with its principal function being to catalyze production of the second messenger, cyclic guanosine monophosphate (cGMP).^{3,4} In addition, NO is known to be involved in neurotransmitter (including dopamine) release, neural development, regeneration, regulation of gene expression, and synaptic plasticity, which is believed to be related to learning and memory.^{5–9}

NO may play a role not only in such physiologic neuronal functions, but also in a variety of neurological disorders in which excessive production of NO leads to neural injury, as NO is one of the oxyradicals. Increasing evidence suggests that oxyradical-mediated CNS neuronal dysfunction is involved in the pathophysiology of schizophrenia. Antioxidant enzyme levels have been shown to be altered in the red blood cells (RBC) of patients with schizophrenia.^{10,11} Lipid peroxidation, which is produced when free radicals damage

membranes or free lipids, is known to be increased in the cerebrospinal fluid (CSF) and in the plasma of patients with schizophrenia.¹² Membrane polyunsaturated fatty acids (PUFAs), which are oxyradical sensitive, are known to be reduced in the brain and RBC membranes in patients with schizophrenia.¹³

The three known isoforms of NO synthase (NOS) responsible for NO production are neuronal (NOS1), inducible (NOS2), and endothelial (NOS3).^{14–17} Production of neuronal NO from L-arginine is catalyzed by NOS1, is Ca²⁺-dependent,^{18,19} and is stimulated by activation of N-methyl-D-aspartate (NMDA) receptors that allow for the influx of Ca²⁺.^{9,20}

The human NOS1 gene has been found to be located on chromosome 12q24.²¹ Recently, a new single nucleotide polymorphism (SNP), a C→T transition located 276 base pairs (bp) downstream from the translation termination site, has been identified in exon 29 of the human NOS1 gene.²² Although linkage studies do not suggest that the NOS1 gene is located within a potential susceptibility region, the physiological findings suggest that the NOS1 deserves investigation as a potential candidate gene in a genesis that may confer an increased susceptibility to schizophrenia. In the present study, we report the results of a case-control study performed to examine if there is an association between a polymorphism in the NOS1 gene and schizophrenia.

In order to assess the ascertainment, namely, to test

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whether the cases and controls in this study were randomly sampled from a population, the Hardy–Weinberg equilibrium was examined. The genotype counts of the T/C in exon 29 both in patients ($\chi^2 = 1.42$, $df = 1$, $P = 0.23$) and in controls ($\chi^2 = 2.19$, $df = 1$, $P = 0.14$) did not deviate significantly from those predicted by the Hardy–Weinberg equilibrium.

The genotype distribution and allele frequencies of the T/C in exon 29 are given in Table 1. There was a significant difference in the genotype distribution between patients and controls ($\chi^2 = 18.03$, $df = 2$, $P = 0.00122$). Furthermore, there was a significant difference in the allele frequencies between patients and controls ($\chi^2 = 20.10$, $df = 1$, $P = 0.000007$; relative risk = 1.92; 95% confidence interval = 1.44–2.55).

We found a significant association between the presence of schizophrenia in Japanese and a polymorphism in exon 29 of the NOS1 gene. The allelic association reported here supports the hypothesis that the NOS1 gene may play a role in the pathophysiology of schizophrenia. This variant, however, is located in the 3'-untranslated region (3'-UTR) of exon 29, and does not result in amino acid substitution. It is still possible that a non-coding alteration may affect splicing, transcription, the efficiency of translation, and protein sequence, as well as mRNA transcript generation, stability, processing or subcellular targeting. Given that: (1) NOS1 mRNA is highly diverse; (2) much of this diversity is restricted to the untranslated regions of the mRNA transcript and may affect its translation or stability; and (3) the 3'-UTR of exon 29 has been shown to affect the size of NOS1 mRNA,^{23,24} this polymorphism may affect the function of the NOS1 gene via NOS1 mRNA diversity. In addition, it is also possible that this polymorphism may be in linkage disequilibrium (LD) with a nearby mutation that could affect any of the above-mentioned processes. Martin *et al*²⁵ have reported that SNPs spanning 40 kb on either side of apolipoprotein E (APOE), an established susceptibility gene for late-onset Alzheimer's disease (AD), is significantly associated with AD, demonstrating the degree of LD between the marker and the functional variant. Furthermore, the putative nearby mutation may be in the NOS1 gene, or in a separate nearby gene.

Increasing evidence suggests that patients with schizophrenia are under increased oxidative stress and decreased antioxidant protection.^{26–29} Increased lipid

peroxidation products in plasma and CSF, and altered levels of both enzymatic and non-enzymatic antioxidants in chronic and drug-naïve first-episode patients with schizophrenia have been reported.³⁰ Altered levels of superoxide dismutase (SOD), an antioxidant enzyme, of RBC in patients with schizophrenia have been reported with levels being either lower¹¹ or higher³¹ than in normal controls. In addition, some evidence suggests that the pathophysiology of schizophrenia is related to activation of the inflammatory response system (IRS), as indicated by increased serum concentrations of interleukin-6 (IL-6), IL-6 receptor (IL-6R), IL-1R antagonist (IL-1RA), and IL-2R and lower serum concentrations of Clara cell secretory protein (CC16), an endogenous anti-inflammatory protein with immunosuppressive and anti-inflammatory effects.³² These results indicate that oxidative stress mediated by active nitrogen species released from inflammatory cells may be involved in the pathophysiology of schizophrenia. Additionally, NOS1 knockout mice showed a lack of phencyclidine (PCP)-induced effects (an animal model of schizophrenia),³³ suggesting that an intact NOS1 system is necessary to obtain PCP-induced effects, implicating NOS1 as a viable drug target in the treatment of schizophrenia. Furthermore, certain aspects of catecholamine signaling in neurons that involve redox systems and synaptic plasticity are presented as the novel 'redox hypothesis' for schizophrenia.³⁴ This hypothesis emphasizes the formation of new synapses and the removal of old ones (synaptic plasticity), as the pathogenesis of schizophrenia, which is modulated in part by the redox balance at the synapse between reactive oxygen species (ROS) (such as hydrogen peroxide and the NO radical) and neuroprotective antioxidants (such as ascorbate, glutathione, and catecholamines). Catecholamines, in particular dopamine, have powerful antioxidant properties and may contribute to synaptic growth and reinforcement-directed learning.³⁴ On the other hand, catecholamines are easily oxidized to toxic quinones on the neuromelanin pathway,³⁴ and it has been hypothesized that oxidative stress mediated by catecholamine orthoquinones (*o*-quinones) may be involved in some of the demonstrated cellular damage in the schizophrenic brain.³⁵

When oxyradicals, including NO, are generated in excess or the cellular antioxidant defense system is

Table 1 Genotype and allele frequencies of C/T in exon 29 of the NOS1 gene in patients with schizophrenia and controls

		Genotypes ^a			Allele frequencies ^b	
		CC	CT	TT	C	T
Schizophrenia	(n = 215)	28	88	99	144 (33.5%)	286 (66.5%)
Controls	(n = 182)	49	81	52	179 (49.2%)	185 (50.8%)

^aSignificant difference in the genotype distribution between patients and controls ($\chi^2 = 18.03$, $df = 2$, $P = 0.00122$).

^bSignificant difference in the allele frequencies between patients and controls ($\chi^2 = 20.10$, $df = 1$, $P = 0.000007$; relative risk = 1.92; 95% confidence interval = 1.44–2.55).

defective, neural injury can result.⁶ The NO radical ($\cdot\text{NO}$) reacts rapidly with O_2 or hydrogen peroxide (H_2O_2) in cells and tissues, yielding higher oxides of nitrogen such as peroxynitrite (ONOO^-). In turn, ONOO^- can act as a neurotoxin, causing cellular dysfunction and even death. Thus, oxyradicals have been implicated in the pathophysiology of neural disease such as Huntington's disease, Alzheimer's disease, and vascular stroke.⁶ In psychiatric disorders, the role of oxidative stress has been considered not only in the pathogenesis of schizophrenia, but also in complications of neuroleptic treatment such as that for tardive dyskinesia (TD).²⁶ We have recently reported a significant association between a manganese superoxide dismutase (MnSOD; a key enzyme of detoxification of superoxide radicals) gene polymorphism and TD.³⁶

In schizophrenic brains, altered numbers of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd)-staining neurons, which are positive for NOS1,³⁷ have been reported in different areas. Patients with schizophrenia have been found to have increased numbers of NADPHd-staining neurons in the pedunclopontine nucleus (PPN), which modulates both thalamocortical neurotransmission and brainstem dopamine activity.^{38,39} Levels of NADPHd-staining neurons in patients with schizophrenia have also been found to be increased in deep cortical and subcortical structures, suggestive of an abnormality in cortical development.^{40,41}

Taking these neurophysiological, neurotoxic, and neuroanatomical findings together with the present genetic findings, the NOS1 gene appears to be a viable candidate gene related to susceptibility to schizophrenia. Further genetic studies with other polymorphic markers or mutations within the NOS1 gene are needed to support our data suggesting an association between the NOS1 gene and schizophrenia.

Methods

Subjects

A total of 215 Japanese patients with schizophrenia (106 males, 109 females, age 52.66 ± 11.74 years, mean \pm SD) participated in this study. Assessment for diagnosis of schizophrenia using DSM-IV criteria was performed by four psychiatrists with consensus, and was based on cross-sectional interviews and case records. None of the subjects had significant neurological comorbidity, epilepsy, mental retardation, or history of substance abuse.

In addition, 182 healthy volunteers (92 males, 90 females, age 54.69 ± 6.48 years, mean \pm SD) were recruited primarily from medical staff as control subjects. Only unaffected subjects whose first- and second-degree relatives had no history of schizophrenia or other psychiatric disorders were included in this study. All controls were over 45 years of age because of the relatively low frequency of schizophrenia after age 45.

All patients had been admitted to one of five hospitals within a 30-km radius of the University of Occu-

pational and Environmental Health; the controls also lived within this area. All subjects in this study were unrelated Japanese originally from the northern part of Kyushu Island, Japan. Informed consent was a premise for participation, and this study was approved by the Ethics Committee of the University of Occupational and Environmental Health.

Genetic analysis

Venous blood samples were collected in EDTA-lined tubes, and DNA was extracted from peripheral leukocytes according to the standard method. The region of interest, as detailed below, was amplified by the polymerase chain reaction (PCR) in a total volume of 20 μl solution containing 100 ng genomic DNA, 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl_2 , 200 μM dNTPs, 20 μM of each primer, and 1 U of Taq DNA polymerase (Roche, Mannheim, Germany) for 40 cycles (94°C for 1 min, 50.5°C for 1 min, and 72°C for 1 min and 30 s). The primers used were: 5'-ACTCCTTGA GTTTCCTGCTGCGATG-3' and 5'-CCATGTTCCAG TGTTTCATGCACAC-3'. The authors introduced a mismatched base into one of the primers (underlined) in order to introduce a restriction site (*Eco*72I restriction enzyme site) into the PCR product when the C allele was present. The PCR products (128 bp) were digested with *Eco*72I at 37°C and then electrophoresed on 3% agarose gels stained with ethidium bromide to detect the C \rightarrow T transition located 276 base pairs (bp) downstream from the translation termination site in exon 29 of the human NOS1 gene. The polymorphism showed a biallelic system, T/C. The C allele showed DNA fragments of 100 bp and 28 bp, whereas the T-allele PCR products remained uncut, with a DNA fragment of 128 bp. Figure 1 shows the representative gel, which exhibits three polymorphic patterns (C/C, C/T, and T/T).

Statistical analysis

The fitness of genotypic distribution to the Hardy-Weinberg equilibrium was analyzed by the χ^2 goodness-of-fit test. Differences in the allele and genotype frequency distribution between patients and controls were evaluated by the χ^2 test.

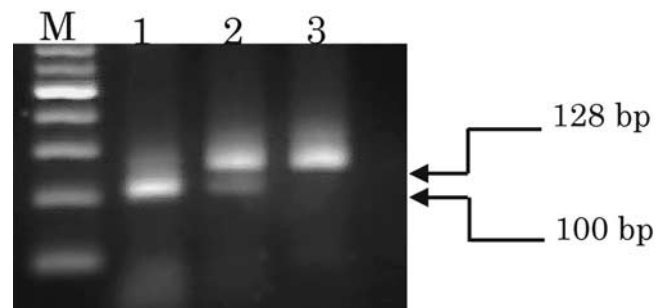


Figure 1 PCR-based genotyping of the *Eco*72I polymorphism of C/T in Exon 29 of NOS1 gene. Lane 1, homozygote of C/C; lane 2, heterozygote of C/T; lane 3, homozygote of T/T. Lane M is a 50-bp DNA ladder marker.

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