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ASSOCIATION ANALYSIS FOR NEURONAL NITRIC OXIDE SYNTHASE GENE POLYMORPHISM WITH PLASMA NITRITE/NITRATE CONCENTRATION IN SCHIZOPHRENIA

ANALIZA ASOCIRANOSTI POLIMORFIZMA GENA ZA NEURONALNU AZOT-MONOKSID SINTAZU I KONCENTRACIJE NITRITA/NITRATA U PLAZMI KOD OBOLELIH OD SHIZOFRENIJE

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Summary

Background: Single nucleotide polymorphisms (SNP) of many genes, including the gene for neuronal nitric oxide synthase (*NOS1*), were found significantly associated with schizophrenia. According to our previously published results of increased plasma nitric oxide concentration in patients with schizophrenia, we hypothesized that the *NOS1* gene polymorphism might be a cause of increased nitric oxide production in patients with schizophrenia and tested the interdependence between plasma nitrite/nitrate concentrations and SNP (a CT transition located in exon 29) of the human *NOS1* gene.
Methods: Nitrite/nitrate concentration was measured in blood plasma of 38 patients with schizophrenia and of 39 age and gender matched healthy persons by the colorimetric test. The *NOS1* gene polymorphism was determined by polymerase chain reaction analysis.
Results: A significantly higher plasma nitrite/nitrate concentration was found in patients with schizophrenia (97.5±33.3 μmol/L, $p<0.001$) in comparison with controls (61.4±18.9 μmol/L). No T/T genotype was found in healthy individuals and there was a significant difference in the genotype distribution between patients and controls ($\chi^2=24.54$, $p=0.0000047$). Furthermore, a significant difference in the

Kratak sadržaj

Uvod: Postoje podaci o značajnoj asocijaciji polimorfizma pojedinačnih nukleotida (SNP) mnogih gena uključujući i gen za neuronalnu azot-monoksid sintazu (*NOS1*) i shizofrenije. Prema našim ranije publikovanim rezultatima koji se odnose na povećanu koncentraciju azot-monoksida u plazmi pacijenata sa šizofrenijom, postavili smo hipotezu da bi polimorfizam *NOS1* gena mogao biti razlog povećane produkcije azot-monoksida kod pacijenata sa shizofrenijom, i stoga testirali međuzavisnost koncentracije nitrita/nitrata i SNP (CT tranzicija u egzonu 29) humanog *NOS1* gena.
Metode: Koncentracija nitrita/nitrata merena je kolorimetrijskim testom u krvnoj plazmi 39 pacijenata sa šizofrenijom i 39 zdravih osoba odgovarajuće starosti i pola. Polimorfizam gena za *NOS1* ispitivan je analizom lančane reakcije polimeraze.
Rezultati: Značajno veće koncentracije nitrita/nitrata u plazmi nađene su kod pacijenata sa shizofrenijom (97,5±33,3 μmol/L, $p<0,001$) u poređenju sa kontrolom (61,4±18,9 μmol/L). Genotip T/T nije nađen kod zdravih osoba, a postojala je značajna razlika u distribuciji genotipa ($\chi^2=24,54$, $p=0,0000047$) i frekvenciji alela ($\chi^2=19,00$, $p<0,000013$, OR=4,45, 95% CI=2,12–9,39) između pacijenata i kontrolne grupe. Takođe, zapažena je značajna

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allele frequencies between patients and controls (χ^2=19.00, p<0.000013, OR=4.45, 95% CI=2.12–9.39) was noted. Also, a significant difference in plasma nitrite/nitrate concentration was observed between patients having the C/T genotype (99.97±33.83 μmol/L) and the corresponding control (C/T) subgroup (63.88±10.26 μmol/L, p<0.01). However, there were no significant differences in nitrite/nitrate concentration between the patient subgroups with different genotypes (C/C, C/T, T/T).
Conclusions: CT transition located in exon 29 of the human *NOS1* gene may be responsible for the increased plasma nitrite/nitrate levels.

Keywords: nitrite/nitrate, nitric oxide synthase, single nucleotide polymorphism, CT transition, schizophrenia

razlika u koncentraciji nitrita/nitrata između pacijenata koji su imali C/T genotip (99,97±33,83 μmol/L) i odgovarajuće kontrolne (C/T) podgrupe (63,88±10,26 μmol/L, p<0,01). Međutim, nije bilo značajne razlike u koncentraciji nitrita/nitrata između podgrupa pacijenata sa različitim genotipovima (C/C, C/T, T/T).
Zaključak: CT tranzicija u egzonu 29 humanog *NOS1* gena može biti razlog povećanog nivoa nitrita/nitrata u plazmi pacijenata.

Ključne reči: nitriti/nitrati, azot-monoksid sintaza, polimorfizam pojedinačnih nukleotida, CT tranzicija, shizofrenija

Introduction

Nitric oxide (NO) is one of the most important signaling molecules which regulates a number of cellular events in the cardiovascular, immune and nervous systems. NO regulates five essential processes in the human body including vascular tone, coagulation, inflammation, oxidation and apoptosis, acting as a hormone, neurotransmitter, paracrine messenger, mediator, cytoprotective and cytotoxic molecule. In the central nervous system (CNS), NO acts as the second messenger of the N-methyl-D-aspartate (NMDA) receptor and interacts with both the dopaminergic and the serotonergic systems. Generally, NO activates the receptor soluble guanilate cyclase by binding to it (1), which leads to increased synthesis of the second messenger, cGMP, which in turn activates cGMP-dependent kinases in target cells. Neuronal nitric oxide synthase (NOS1) is connected to NMDA receptors whose activation increases NO production (2). Endogenously produced NO around NMDA synapses reflects the activity of glutamate-mediated neurotransmission (3). In addition, NO is known to have effects on the storage, uptake and/or release of most other neurotransmitters in the CNS including acetylcholine, dopamine, noradrenaline, GABA, taurine and glycine that have all been implicated in schizophrenia (4). Further, NO is a diffusible molecule which may react with extrasynaptic receptors at target cell membranes at a distance from the site of its synthesis (5) and take part in nonsynaptic communication processes. Its production in the CNS is associated with the cognitive function, the induction and maintenance of synaptic plasticity, neural development, regeneration, regulation of gene expression, the control of sleep, appetite, body temperature and neurosecretion (6, 7). As a free radical, NO may have a toxic effect at higher concentrations. The NO-mediated cytotoxicity is due to its conversion into peroxynitrite in a reaction with superoxide. Peroxynitrite can react with a wide range of biological molecules leading to enzyme inhibition and autooxidation of the neurotransmitter dopamine. The chemical interaction of dopamine and its metabolites with NO constitutes a source of neurotoxic molecules of relevance to neuropsychiatric disorders (8). Given the broad range of functions of NO, it seems to be a promising candidate molecule in the pathogenesis of endogenous psychoses, including schizophrenia. This is in accordance with finding of significantly increased plasma NO concentration in patients with schizophrenia in comparison with healthy controls (9).

In the CNS, NO is produced from the amino acid L-arginine by two isoforms of nitric oxide synthase (NOS), by neuronal NOS (NOS1) and by endothelial NOS (NOS3). NOS1 is the major NOS isoform, accounting for about 90% of the overall NO production (10). The second source of NO is NOS3, which may be beneficial in that it protects from cerebral ischemia through vasodilation as well as the inhibition of leukocyte adhesion and platelet aggregation (11). Although endothelium derived NO has been identified as a major player in stroke and ischemia, its role in neuropsychiatric disorders is less clear. Since it is known that the regulation of cerebral blood flow is altered in schizophrenia (12), the influence of NO on this physiological process may be significant.

Schizophrenia has a substantial genetic background, with a heritability of up to 81% (13). Many genes have been examined as candidate genes, but no functional gene variant or mutation have yet been derived from linkage analyses. The analysis of the mini-haplotype of *NOS1* revealed a significant association with schizophrenia, and single-marker association analysis showed that the exon 1c promoter polymorphism was linked to schizophrenia, suggesting that regulatory rather than coding variants of *NOS1* contribute to the genetic risk for schizophrenia (14). In addition, Shinkai et al. (15) showed that the single nucleotide polymorphism (SNP), a CT transition located 276 base pairs (bp) downstream from the translation termination site, identified in exon 29 of the human *NOS1* gene, is significantly associated with schizophrenia, suggesting that the *NOS1* gene may play a role in the pathophysiology of schizophrenia. On the basis of this finding and our results related to the increased plasma NO concentrations in patients with schizophrenia (9) in this study, we report the results of a case–control study performed to examine

if there is any association between the polymorphism C276T and increased plasma NO concentration in schizophrenia.

Materials and Methods

Subjects recruitment and assessment

This study included 38 patients with schizophrenia (22 males, 16 females, age 32.7±9.4 years, mean±SD) recruited at the Clinic of Psychiatry and the Clinic for Mental Health Protection of the Clinical Centre Niš. Assessment for diagnosis of schizophrenia using the DMS-IV criteria was performed by two psychiatrists with consensus, and was based on cross-sectional interviews and case records using the SCID (Structured Clinical Interview for DSM-IV). Disease evaluation and clinical management of patients were performed using the PANSS for scoring positive symptoms, negative symptoms and the general psychopathology scale which presented the structure of clinical disease manifestation. Heredity was present in 15 of the 38 schizophrenics. None of the subjects had significant neurological comorbidity, epilepsy, mental retardation, a history of substance abuse or immune, inflammatory, liver and vascular diseases.

The study also included 39 healthy volunteers (18 males, 21 females, age 30.9±6.9 years, mean±SD) recruited from the medical staff as control subjects. The subjects whose first- and second-degree relatives had a history of schizophrenia or other psychiatric disorders were excluded from the study. The patients and controls were matched according to age, gender, living conditions, living settings and habits.

All the participants in this study were unrelated Serbs originally from the southeast part of Serbia. All the subjects provided written informed consent, and the study was approved by the Clinical Centre Niš Ethics Committee and carried out in accordance with *The Code of Ethics* of the World Medical Association for experiments involving humans.

Determination of plasma nitrite/nitrate (NO_2^-/NO_3^-) concentration

Venous blood was collected in two vacutainer tubes containing potassium EDTA as an anticoagulant. From one tube series, plasma samples were separated and stored at –20 °C until the measurement of NO_2^-/NO_3^-. The concentrations of NO_2^-/NO_3^- were measured using the modified cadmium-reduction method of Navaro-Gonzalvez et al. (16) based on the Griss reaction.

Genetic analysis

The second series of EDTA tubes was used for DNA extraction from peripheral leukocytes, which was performed using a commercial kit (Fermentas Thermo Fischer Scientific Inc). The region of interest was amplified by the polymerase chain reaction (PCR) in a total volume of 25 μL solution containing 12.5 μL Kappa Mix (buffer, $MgCl_2$, dNTP, TaqPolimerase in 0.5 μL each), 10 pmol of the primers for the amplification of the *NOS1* gene sequence, 1 μL of isolated DNA and ultra distillated water up to 25 μL. The primers used were: 5'-ACTCCTTGAGTTTTCCTGCTGCGATG-3' and 5'-CCATGTTCCAGTGGTTTCATGCACAC-3' (15). The conditions for amplification were as follows: 94 °C for 1 min, 94 °C for 30'' (35x), 55 °C for 30'' (35x), 72 °C for 1 min (35x), 72 °C for 7 min Taq polymerase, 94 °C for 5 min, 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. After that, the digestion of PCR products was performed by the restriction enzyme Eco72I (Fermentas, Thermo Fischer Scientific Inc), according to the instructions of the manufacturer. Electrophoretic separation of the DNA fragments obtained on 3% agarose gels stained with ethidium bromide to detect (the bands) CT transition located 276 base pairs (bp) downstream from the translation termination site in exon 29 of the human *NOS1* gene was evaluated. The polymorphism showed a biallelic system, C/T. The C allele showed DNA fragments of 100 bp and 28 bp, T-allele (homozygous) PCR products showed only one DNA fragment of 128 bp, whereas heterozygous C/T showed three fragments of 128 bp, 100 bp and 28 bp.

Statistical analysis

Data analysis was performed using SigmaStat and StatCalc computer programs. The differences in NO_2^-/NO_3^- concentrations between the groups were tested by the One Way Analysis of Variance. The differences in the allele and genotype frequency distribution between the patients and controls were evaluated by χ^2 test and Allelic wise association test.

Results

The mean plasma NO_2^-/NO_3^- concentration was significantly higher in patients with schizophrenia

Table I Demographic characteristics and plasma NO^{2-}/NO^{3-} concentrations in patients with schizophrenia and healthy controls.

	Control	Schizophrenia
Total (n)	39	38
Male/female (n)	18/21	22/16
Age (years)	30.9±6.9	32.7±9.4
Heredity (+/–)	–	15/23
NO_2^-/NO_3^- (μmol/L)	61.4±18.9	97.5±33.3*

* - $p<0.001$ vs. Controls

Table II Genotype distribution of C/T in exon 29 of the *NOS1* gene in patients with schizophrenia and controls.

Group	C/C	C/T	T/T
Control	19 (48.7%)	20 (51.3%)	0
Schizophrenia	2 (5.3%)**	26 (68.4%)	10 (26.3%)*

χ^2=24.54, p=0.00000470 between the groups

*χ^2=11.64, p=0.0004309 vs. Controls

**χ^2=18.09, p=0.0000211, OR=0.06, 95% CI=0.01–0.30 vs. Controls

Table III Allele frequencies of C/T in exon 29 of the *NOS1* gene in patients with schizophrenia and controls.

Group	C	T
Control	58 (74.4%)	20 (25.6%)
Schizophrenia	30 (39.5%)	46 (60.5%)*

*χ^2=19.00, p=0.000013, OR=4.45, 95% CI=2.12–9.39 vs. Controls

Table IV Genotype distribution of C/T in exon 29 of the *NOS1* gene according to the sex of the studied individuals.

Group	Sex	C/C	C/T	T/T
Control	m	9 (23.1%)*	9 (23.1%)	0
	f	10 (25.6%)**	11 (28.2%)	0
Schizophrenia	m	2 (5.3%)	15 (39.5%)	5 (13.2%)***
	f	0	11 (28.9%)	5 (13.2%)****

* χ^2=8.10, p=0.0055935 vs. shizophrenic males

** χ^2=10.16, p=0.0017253 vs. schizophrenic females

*** χ^2=4.56, p=0.0400208 vs. control males

**** χ^2=5.54, p=0.0478424 vs. control females

Table V Plasma NO^{2-}/NO^{3-} concentrations in patients with schizophrenia and controls with different genotypes.

NO_2^-/NO_3^- (μmol/L)	C/C	C/T	T/T
Control group	69.64±9.09	63.88±10.26	–
Schizophrenia group	84.80±33.80	99.97±33.83[A]	88.17±29.37

A – p<0.01 vs. Controls

(97.5±33.3 μmol/L, p<0.001) in comparison with healthy controls (61.4±18.9 μmol/L) (*Table I*). No significant difference was found between the patient groups with positive and negative heredity, between patients with different PANSS scores or between patients treated with the first generation antipsychotics and second generation antipsychotics, respectively.

The genotype distribution (C/C, C/T, T/T) in exon 29 of human *NOS1* (*Table II*) showed a significant difference between patients and controls (χ^2=24.54, p=0.0000047). Heterozygous C/T (68.4%) was predominant in the patient group. The genotype T/T was present in 26.3% and homozygous C/C in only 5.35% of the patients. Homozygous T/T was not observed in the healthy controls, while the genotype C/C (48.7%) and heterozygous C/T (51.3%) were found in similar percentages. Significantly higher frequency of the T/T genotype (χ^2=11.64, p=0.0004309) and significantly lower frequency of the C/C genotype (χ^2=18.09, p=0.0000211, OR=0.06, 95% CI=0.01–0.30) were found in the patient group in comparison with controls. Furthermore, there was a significant difference in allele frequencies between the patients and controls (χ^2=19.00, p=0.000013, OR=4.45, 95% CI=2.12–9.39) (*Table III*). In the healthy controls, the genotypes C/C and C/T showed similar distribution in females and males. The C/C genotype was not noted in schizophrenic females, while the T/T genotype was equally present in males and females (*Table IV*). Significantly higher frequency of the C/C genotype was found in control males (χ^2=8.10, p=0.0055935) and females (χ^2=10.16, p=0.0017253; Fisher exact test) compared to the schizophrenic ones. T allele is present in a significantly higher percentage in patient males and females than in the corresponding groups of controls (χ^2=8.10, p=0.0044260, OR=3.95, 95% CI=1.37–11.64, and χ^2=13.41, p=0.0002498, OR=6.58, 95% CI=2.08–21.57, respectively; Mantel-Haeszel). Allele frequencies were also tested by the Allelic wise association test showing χ^2=0.51789, df=1. Although all the patient subgroups with different genotypes showed higher NO_2^-/NO_3^- levels, a significant difference was found only between the patients with the C/T genotype (99.9±33.83 μmol/L) and the corresponding control (C/T) subgroup (63.88±10.26 μmol/L, p<0.01). However, there was no significant difference in NO_2^-/NO_3^- values between the patient subgroups with different genotypes (*Table V*), or between the groups with different alleles.

Discussion

Despite the intensive research being conducted to identify specific biological markers of schizophrenia, no unique or indicative marker of this disease has been recognized. A substantial amount of data suggested NO as a promising molecule, because many processes in the brain are linked to this signaling molecule. Many research results, including our own, showed that the metabolism of NO is strongly disturbed in patients with schizophrenia. However, the

results related to NO in schizophrenia have been inconsistent. Ramirez et al. (17) reported diminished levels of the NO metabolites, nitrite and nitrate, in the CSF of schizophrenics, suggesting that, in the brain, there was a reduction of NO formation in schizophrenia. Decreased levels of the NO metabolites were found in the plasma and polymorphonuclear blood cells of schizophrenics. On the other hand, increased NO levels were reported in plasma, serum, erythrocytes and some brain regions (4).

A highly significant increase in plasma NO metabolites that we found in schizophrenics in repeated studies suggests that NO may exert toxic effects (18–21). But, the reason for this increased NO production stays unclear. Shinkai et al. (15) showed that there was a significant association between the presence of schizophrenia in the Japanese and a single nucleotide polymorphism on chromosome 12q24 (C→T transition located 276 base pairs downstream from the translation termination site). According to their hypothesis, the *NOS1* gene may play a role in the pathophysiology of schizophrenia, despite the fact that this variant is located in the 3'-untranslated region of exon 29 and does not result in amino acid substitution. A noncoding alteration may affect splicing, transcription, the efficiency of translation and protein sequence, as well as mRNA transcript generation, stability, processing or subcellular targeting. Also, the 3'-UTR of exon 29 has been shown to affect the function of NOS1 mRNA (22). So, the above mentioned polymorphism may affect the function of the *NOS1* gene via NOS1 mRNA diversity.

Although our study was limited by the small number of patients with schizophrenia, we also noted a significant association of the T/T genotype frequency in exon 29 of *NOS1* with schizophrenia. It is present in more than one fourth of the patients. Furthermore, none of the healthy controls had homozygous T/T, and this might be a consequence of the small number of patients. In addition, no significant differences were found between the concentrations of nitric oxide metabolites in patients with different genotypes (C/C, C/T, T/T). However, the significant difference in NO concentration between the patient group with the C/T genotype and the corresponding control group suggests that T allele may be responsible for an increased NO concentration in the patient group. According to the findings of Silberberg et al. (23), the overexpression of specific NOS1 isoforms ('NOS1_1d' and 'NOS1_1f'), which is unique to schizophrenia, may be responsible for the increased NO production. The studies related to the *NOS1* polymorphism in the drug-treated patients with schizophrenia who developed tardive dyskinesia did not find any support in genetic analyses (24, 25). Okumura et al. (26) could not replicate the association between seven SNPs in *NOS1* and schizophrenia found in several previously reported studies. However, two independent studies that analyzed SNP within the CAPON (the carboxyl-terminal PDZ-ligand of *NOS1*) provided evidence of significant linkage disequilibrium in schizophrenia (27, 28).

In the genome-wide association study (GWAS) on schizophrenia (29), a UK-sample of 479 cases with schizophrenia was genotyped in comparison to control subjects with follow up of 12 putative loci in international replication sets of approximately 15,000 cases and controls. In these cohorts and a combined bipolar and schizophrenia UK-sample, six SNPs (including rs6490121 at the *NOS1* locus) supported the association, with the strongest evidence for SNP-marker rs1344706 at the zinc finger *ZNF804A* locus on chromosome 2q32.1. Schanze et al. (30) attempted replication of these findings in a German population of 2,154 individuals (632 with affective disorders, 937 with schizophrenia, and 585 controls), but found none of the GWAS risk alleles significantly associated with psychosis.

Having in mind that increased serum concentrations of interleukin-6 (IL-6), IL-6 receptor (IL-6R), IL-1R antagonist (IL-1RA) and IL-2R were observed in patients with schizophrenia (31), another source of increased NO production may be immune and inflammatory cells. Thus, oxidative stress mediated by active nitrogen species as well as oxygen species released from inflammatory cells may be involved in the pathophysiology of schizophrenia. Increasing evidence suggests the existence of oxidative stress in schizophrenia as a consequence of altered both enzymatic and nonenzymatic antioxidants in chronic and drug-naïve patients (32–35). An upregulated production of NO by reactive astrocytes was demonstrated in Alzheimer's disease of humans (36) and in an animal model of Alzheimer's disease (37). In mice lacking *NOS1*, the process of demyelination is greatly prevented (38), and *NOS1* knockout mice show a lack of phencyclidine-induced effects (an animal model of schizophrenia) (39). All these findings support the hypothesis that *NOS1* plays an important role in both neurodegeneration and schizophrenic psychosis and further studies are needed to identify the precise mechanism of its action.

In conclusion, our results confirm the association of *NOS1* gene polymorphism with schizophrenia and suggest that T allele may be responsible for the significant increase in patient plasma NO concentrations.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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