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CLINICAL STUDY

eNOS and XRCC4 VNTR variants contribute to formation of nicotine dependence and/or schizophrenia

Pehlivan S[1], Uysal MA[2], Aydin PC[3], Pehlivan M[4], Nursal AF[5], Yavuzlar H[3], Kurnaz S[1], Sever U[1], Yavuz FK[2], Uysal S[2], Aydin N[2]

Department of Medical Biology, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey.
sacide.pehlivan@istanbul.edu.tr

ABSTRACT
BACKGROUND: This study aimed to evaluate whether VNTR variants in the Endothelial Nitric Oxide Synthase (eNOS) and the XRCC4 gene play any role in nicotine dependence (ND) and/or Schizophrenia+ND (Sch+ND) ethiopathogenesis.
METHODS: Present study included 100 individuals with ND, 60 patients with Sch+ND, and 70 healthy controls. These variants were analyzed using PCR.
RESULTS: The cases with ND had higher eNOS VNTR-BB genotype than the healthy control subjects (p = 0.001). eNOS-AA genotype was lower in cases with Sch+ND and ND groups compared to the controls (p = 0.001, p = 0.001, respectively). eNOS-B allele was found significantly more frequently in Sch+ND group compared to the controls (p = 0.001). eNOS-A allele was significantly lower in ND group than the controls (p = 0.001). XRCC4-ID genotype was more common in the ND group than the control group (p = 0.001) as heterozygosity disadvantage. XRCC4-DD genotype was more common in the Sch+ND group compared to the controls (p = 0.035). The frequency of XRCC4-I allele was lower in the Sch+ND group compared to the controls (p = 0.012).
CONCLUSIONS: Our results showed that eNOS and XRCC4 VNTR variants might play a potential role in Sch+ND and/or ND pathophysiology *(Tab. 2, Ref. 48)*. Text in PDF *www.elis.sk.*
KEY WORDS: schizophrenia, endothelial nitric oxide synthase, XRCC4, nicotine dependence.

Introduction

Schizophrenia (Sch) is a complex psychiatric disorder, which affects approximately 1 % of the general population (1). This disease is characterized by various symptoms and the prognosis and outcome of the disease differ among the patients (1). Until recently, ethiopathology of Sch has remained unclear. Distortions of several neurotransmitter systems, most notably the dopamine, glutamate, cholinergic, the serotonergic and the γ-aminobutyric acid (GABA) systems are believed to be crucial for the occurrence of this disease (2). Nicotine dependence (ND) is a serious public health issue leading to millions of preventable deaths worldwide.

Nitric oxide (NO) is a soluble, short-lived and freely diffusible gas, which has been reported to conduct numerous signaling tasks throughout the organism such as the central and peripheral nervous system (3). NO is produced after activation of glutamate receptors, mainly N-methyl-D aspatate (NMDA) subtype. NO is particularly crucial as the secondary messenger of NMDA receptor activation, which interacts with both dopaminergic and serotonergic pathways (4). Studies report evidence implying the role of NO in Sch. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Some studies reported that the high concentration of NO in cigarette smoke interferes with the action of enzyme NOS or lowers the level of NOS (5). In rats, NOS inhibitors can alleviate symptoms of the nicotine abstinence syndrome, and co-administration of L-arginine is likely to diminish the attenuating effect of the the NOS inhibitor (6). NOS has three distinct isoforms; neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3) (7). eNOS involves mainly the endothelium, continuously producing NO and it helps to maintain basal vascular tone and cerebral blood flow. The eNOS gene located on chromosome 7q35-36 that has 26 exons that span >21kb of the genome encodes eNOS (GenBank D26607) (8). A variable number of tandem repeat (VNTR) (27 nt) in intron 4 of eNOS gene is responsible for production of more than 25 % of basal plasma NO (9). It was established that this VNTR produces a small RNA (sirRNA from "short intronic repeat small RNA") inhibiting eNOS expression on the transcriptional level (10).

Several exogenous agents lead to DNA double-strand breaks (DSBs), which take place spontaneously during the cell cycle. DNA double-strand breaks (DSBs) are capable of destroying the integrity of the DNA molecule (11). DNA-repair process is crucial for the protection of the genome from environmental dam-

[1]Department of Medical Biology, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey, [2]Department of Chest Diseases, Yedikule Hospital For Chest Diseases and Thoracic Surgery Training and Research Hospital, Istanbul, Turkey, [3]Department of Psychiatry, Bakirkoy Research and Training Hospital for Psychiatry, Neurology and Neurosurgery, Istanbul, Turkey, [4]Department of Haematology, Gaziantep Univesity, Faculty of Medicine, Gaziantep, Turkey, and [5]Department of Medical Genetics, Hitit University, Faculty of Medicine, Corum, Turkey

Address for correspondence: S. Pehlivan, Prof, Dr, Istanbul University, Istanbul Faculty of Medicine
Department of Medical Biology, Capa-Fatih, Istanbul.
Phone: +90.212.6351168

age such as tobacco smoke. Evidence suggests that DNA repair is altered in chronic smokers. Furthermore, dysfunctional DNA damage repair process in patients with Sch has been discussed in numerous studies (12, 13). The gene encoding X-ray repair cross-complementation group4 (XRCC4; OMIM: 194363) play a role in repair of DSBs (14). The protein encoded by XRCC4 is composed of 336 amino acid residues distributed among 8 exons, and has a long helical stem domain, which accounts for multimerization and interaction with DNA ligase IV (15). A VNTR variant exists in intron 3 of the XRCC4 gene.

In the present study, we aimed to find out whether the VNTR variants in eNOS and XRCC4 genes play any role in ND and/or Sch+ND ethiopathogenesis.

Patients and methods

Subjects

This study included 100 individuals with ND (female/male: 46/54), 60 patients with Sch+ND (female/male: 45/15), and 70 healthy controls (female/male: 45/25). The subjects were selected among the individuals from Bakirkoy Research and Training Hospital for Psychiatry Hospital, Istanbul Turkey and Yedikule Hospital For Chest Diseases and Thoracic Surgery Training and Research Hospital, Istanbul Turkey. Sch diagnosis was based on the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) Criteria. The control group was selected from the voluntaries who did not have any psychological disorder and were non-smokers. Both the study and the control groups contained individuals of Turkish origin, and they were all over 18 years of age. Informed written consent was obtained from all patients and subjects before enrollment to the study, according to the ethical guidelines of the Declaration of Helsinki and the investigation was approved by the Instuitional Ethical Committee.

Genotyping

Genomic DNA was extracted from the whole blood treated with EDTA according to the established method (16). The extracted DNA was stored at -20°C until analysis. eNOS (intron 4 VNTR A/B) and XRCC4 (intron 3 VNTR I/D) variants were genotyped by PCR described previously (17, 18) and agarose gel electrophoresis. The experimental process was repeated twice for each sample. The distribution of the genotypes of patients and control subjects was compared.

Statistical analysis

All data were analyzed using software SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL; USA). The statistical significance of the differences between the patient and control groups was estimated by logistic regression analysis. Odds ratio (OR) and 95 % confidence interval (CI) were also calculated. Differences in eNOS and XRCC4 VNTR genotype distribution between the patients and controls were compared with chi-square test and, Fisher's exact test was used when needed. The data were analyzed for appropriateness between the observed and expected genotypes as well as for Hardy-Weinberg Equilibrium (HWE). All analyses were two-tailed, and differences were interpreted as statistically significant when $p < 0.05$.

Results

A total number of 230 subjects from a Turkish population were recruited (100 ND, 60 Sch+ND and 70 healthy controls) in this study. The results of genotype distribution and allele frequency of eNOS VNTR variant in ND group, Sch+ND group versus controls are presented in the Table 1.

eNOS genotyping

A statistically significant difference in both the genotype distribution and allele frequency between cases and healthy controls was found for eNOS VNTR variant. The cases with ND had a higher frequency of eNOS VNTR BB genotype than the healthy control cases (OR: 4.218, 95%Cl: 2.154–8.260; p = 0.001). Frequency of AA genotype was lower in cases of both Sch+ND group and ND group in comparison with the healthy controls, respectively (OR: 6.417, 95%Cl: 2.067–19.923; p = 0.001; OR: 14.819, 95%Cl: 4.225–51.978; p = 0.001, respectively). B allele was found significantly more common in Sch+ND group compared to the controls (OR: 2.768, 95%Cl: 1.591–4.815; p = 0.001). Frequency of A allele of eNOS VNTR was significantly lower in the ND group than the controls (OR: 5.342, 95%Cl: 3.105–9.190; p = 0.001) .

Tab. 1. Comparison of eNOS VNTR variant between cases with Sch+ND, ND and control subjects.

eNOS VNTR	Sch+ND[a]	ND[b]	Controls	OR*	95% CI*	p
Genotypes	n=60 (%)	n=100 (%)	n=70 (%)			
BB	39 (65)	79 (79)	33 (47.1)	0.480 [a] 4.218 [b]	0.237-0.974 [a] 2.154-8.260 [b]	0.052 [a] 0.001 [b]
AB	17 (28.4)	18 (18)	15 (21.4)	0.690 [a] 1.242 [b]	0.310-1.536 [a] 0.578-2.672 [b]	0.417 [a] 0.694 [b]
AA	4 (6.6)	3 (3)	22 (31.5)	6.417 [a] 14.819 [b]	2.067-19.923 [a] 4.225-51.978 [b]	0.001 [a] 0.001 [b]
Alleles						
B	95 (79.2)	176 (88)	81 (57.8)	2.768 [a]	1.591-4.815 [a]	0.001 [a]
A	25 (20.8)	24 (12)	59 (42.2)	5.342 [b]	3.105-9.190 [b]	0.001 [b]
HWEp	0.2745	0.1396	2.730			

Fisher's Exact Test, [a] comparison of genotype frequencies between Sch+ND and healthy control groups; [b] comparison of genotype frequencies between ND and healthy control groups. HWEp: Hardy Weinberg Equliibrium.

Tab. 2. Comparison of XRCC4 VNTR variant between patients with Sch+ND, ND and healthy control subjects.

XRCC4 VNTR	Sch+ND	ND	Controls	OR*	95% CI*	p
Genotypes	n=48 (%)	n=103 (%)	n=69 (%)			
II	13 (27)	21 (20)	24 (35)	1.846 [a] 2.083 [b]	0.837-4.073 [a] 1.045-4.149 [b]	0.170 [a] 0.051 [b]
ID	11 (23)	55 (54)	19 (42)	1.624 [a] 0.332 [b]	0.699-3.771 [a] 0.172-0.638 [b]	0.299 [a] 0.001 [b]
DD	24 (50)	27 (26)	16 (23)	0.428 [a] 0.850 [b]	0.199-0.919 [a] 0.417-1.730 [b]	0.035 [a] 0.721 [b]
Alleles						
I	37 (39)	97 (47)	77 (56)	2.013 [a]	1.184-3.423 [a]	0.012 [a]
D	59 (61)	109 (53)	61 (44)	1.418 [b]	0.920-2.188 [b]	0.124 [b]
HWEp	0.000	0.467	0.008			

Fisher's Exact Test, [a] comparison of genotype frequencies between Sch+ ND and healthy control groups; [b] comparison of genotype frequencies between ND and healthy control groups. HWEp: Hardy Weinberg Equliibrium.

XRCC4 genotyping

Genotype and allele frequencies of XRCC4 gene intron 3 VNTR variant are shown in the Table 2. There was a significant difference for genotype distribution and allele frequency of VNTR variant of XRCC4 gene between groups. XRCC4 ID genotype was more common in ND group than in controls (OR: 0.332, 95%Cl: 0.172–0.638; p = 0.001). The cases with Sch+ND had a higher DD genotype than healthy control group (OR: 0.428, 95%Cl: 0.417–1.730; p = 0.035). Frequency of XRCC4 VNTR variant I allele was lower in the Sch+ND group compared to the healthy group (OR: 2.013, 95%Cl: 1.184–3.423; p =0.012).

Discussion

Sch is a severe psychiatric disease with a chronic course, mostly occurring at a young age. Genetic factors are involved in up to 80 % of cases with Sch (1). Cigarette smoking can be regarded as an easy and common way to receive multiple doses of psychoactive drug nicotine (1). Besides, smoking results in ND and it is the most significant cause of preventable death. There are more than 4000 components in cigarette smoke, but the pharmacological impacts of dependence are primarily due to nicotine, which acts through neuronal nicotinic acetylcholine receptors (nAChRs). It was shown that acute and chronic nicotine administration to rats elevates the levels of stable metabolites of NO in various brain regions (19). The apparently high prevalence of smoking among the patients with Sch indicates the possibility that the common co-occurrence of nicotine use and Sch can be associated with mutual underlying neurobiological factors (20). Several studies showed that NO may act in the pathogenesis of many psychiatric diseases, including depression (21), bipolar disorder (22), and Sch (23, 24). Nakano et al (25) suggested that plasma levels of NO and its metabolites are diminished in patients with Sch and they reported that the baseline plasma NO metabolites levels were significantly lower in the Sch group. Arinole et al (26) showed that plasma NO level was significantly elevated in drug free patients with Sch compared to the controls or treated patients in a Nigerian cohort.

The eNOS 27-bp VNTR variant in intron 4 possesses two common alleles: 4 repeats and 5 repeats. Two less common alleles were found (with 6 and 3 repeats) in African and Colombian populations (27). Some studies suggest that carriers of 4 repeat allele have decreased NO plasma levels and diminished protein expression; however, there is a controversy among the various studies. The variant is probably in linkage disequilibrium with other functional variants in regulatory regions of the eNOS gene (27). The biological influence of the eNOS VNTR variant remains unclear, however it was implied that this variant modulates the expression of eNOS by the production of sirRNAs. Human eNOS is expressed in the cerebral endothelial cells throughout the brain and its mRNA is also expressed in several regions of basal ganglia such as caudate nucleus, putamen, substantia nigra and subthalamicc nucleus (28). The impaired expression of eNOS was believed to be major abnormality common to neurodegenerative diseases. Previously, it was reported that nNOS 276 C+ genotype incidence was significantly higher in the patients with obsessive-compulsive disorder than the controls (29). Also, the genotype distribution of the variants in exon 22 of the iNOSA gene and in exon 29 of the nNOS gene were significantly different between the patients with recurrent depressive disorder and the control cases (30). Reif et al (31) showed that the eNOS genotype might bear a modest genetic risk of developing bipolar disorder. Also, it was shown that three eNOS variants were not significantly different in depressed Japanese patients (32). On the other hand, Zeman et al (33) reported that the eNOS genotype (rs1979983) was not associated with depression. The analysis of the mini-haplotype of nNOS showed a crucial relation with Sch, and single-marker association analysis revealed that the exon 1c promoter variant was related to Sch, indicating that regulatory rather than coding variants of nNOS act as a genetic risk factor for Sch (34). Furthermore, Shinkai et al (35) found that the variant, a CT transition located 276 bp downstream from the translation termination site, described in exon 29 of human nNOS gene, was significantly related with Sch, indicating that the nNOS gene involves in the pathophysiology of Sch. Okumura et al (36) found that two variants (rs3782219 and rs3782206) of nNOS gene showed a significant association with Sch in allele and/or genotype-wise analysis.

In this study, we tested the hypothesis that VNTR variants of eNOS/XRCC4 might influence the development of ND and/or Sch+ND. We found that the cases with ND had a higher eNOS VNTR BB genotype than healthy control (p = 0.001) (Tab. 1). Also, B allele was found in a significantly higher frequency in Sch+ND

group compared to controls (p = 0.001). Frequency of A allele of eNOS VNTR was significantly lower in the ND group than in the control group (p = 0.001). These results supported the idea that eNOS VNTR AA genotype and A allele had a protective effect on the risk of Sch/ND and ND.

DNA damage can either arise from exposure to exogenous DNA damaging agents like tobacco smoke or UV radiation, endogenous sources such as oxidative stress originating from the respiratory chain, or it can be due to decrease in repair of normal levels of DNA damage continuously occurring in our genomes (11, 37). Tobacco smoke has numerous potent chemical carcinogens such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines and N-nitroso compounds. These carcinogens may cause direct and indirect DNA damage. Several types of DNA damage are repaired through multiple repair pathways where numerous proteins take a part. DNA-repair process is very important for the protection of the genome from environmental damage including tobacco smoke. Nucleotide excision repair pathway concerns with DNA damage created by the tobacco-related carcinogen benzo(a) pyrene, while base excision repair pathway repairs DNA damage created by reactive oxygen species resulting from cigarette smoke (37). The signs of enhanced oxidative stress and oxidative DNA damage were found in different tissues of the patients with Sch (38). The mutagens sensitivity and efficacy of DNA repair are affected by variation in many genes such as XRCC genes.

A nuclear phoshoprotein is encoded and multimerized by XRCC4 gene. XRCC4 also interacts with DNA Ligase4 and DNA-dependent protein kinase, involving in the non-homologous end joining (NHEJ) pathway (39). In mice, XRCC4-deficiency leads to massive neuronal apoptosis hence it is embryo-lethal condition. XRCC4 was reported to interact with p53 in the modulation of apoptosis, implying that XRCC4 is essential for maintaining genomic stability and for the suppression of tumors (40). DNA-repairing gene variations might affect genomic instability as well as influencing protein function and growth intervention. Numerous studies have associated the variants in DNA repair genes to the occurrence of Sch. The observation of high smoking prevalence among Sch patients (the rate of smoking among the patients with Sch has been reported to be at least three to five times more than in the general population) gives rise to thought that ND could be an etiological factor for this disease. There are many studies that investigated the association between XRCC gene variants and cancer (41), preeclampsia (42), ulcerative colitis (43), and rheumatoid arthritis (44). Saadat et al (45) showed that the 399Gln allele in exon 10 variant of XRCC1 gene had in increased the risk of Sch. However, it was reported that there was no association between Arg194Trp variant of XRCC1 gene and Sch in the same population (46). Also, it was reported that XRCC1 gene (Arg399Gln) variant Gln399Gln genotype was associated with Sch in South Indian population (47). Odemis et al (48) showed that XRCC1 (Gln) and XRCC3 (Thr) alleles were significantly more frequent among the patients with Sch than the controls.

In the present study, we found that XRCC4 ID genotype was more common in ND group than in the controls (p = 0.001). Besides, it was found that XRCC4 VNTR DD genotype was more common in the Sch+ND group compared to the control group (p = 0.001). The present study is the first research in literature, which demonstrated that DD genotype was associated with susceptibility to Sch and presence of ID genotype had a role as "heterozygosity disadvantage for ND" in Turkish population. It was found that the frequency of XRCC4 I allele was lower in the Sch+ND group than in the control group (p = 0.012). It is possible that XRCC4 I allel can play a role in protecting from this disease.

To our knowledge, the present study is the first report showing a significant association between eNOS/XRCC4 VNTR variants and ND and/or Sch+ND in a Turkish cohort. Our results showed that eNOS/XRCC4 VNTR variants constitute a risk factor for both ND and Sch+ND ethiopathogenesis. However, large-scale studies should be replicated with different subjects and/or other ethnic groups to fully elucidate the effects of these variants on susceptibility to ND and/or Sch+ND.

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