RESEARCH ARTICLE

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Association of Nicotine Use Disorder with Neuroxin 3 Gene Polymorphisms

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SUMMARY

Objective: In this study, we aimed to investigate the Neurexin 3 gene (NRXN3) polymorphisms in the rs 221473, rs 221497, rs1004212 and rs11624704 regions in relation to nicotine use disorder (NUD) in the Turkish population.

Method: Power analysis indicated that the NUD group and the control group of this study should each comprise 200 participants in the 18-65 year age range. The NUD group consisted of individuals without a psychiatric first axis disorder except for NUD, mental retardation, past head trauma or a neurological disorder, who had smoked minimally10 cigarettes per day for at least 1 year. The control group included individuals without a serious chronic physical illness, a previous psychiatric disorder or mental retardation and who responded "no" to the question "have you ever smoked?" A sociodemographic questionnaire and the Fagerström nicotine dependence scale (FNDS) for the NUD group were utilized. Venous blood samples of all participants were taken into tubes containing EDTA (ethylene daimine tetra acetic acid) for DNA extraction. Duplex fluorescence melting curve analysis was used for genotype detection and differentiation. .

Results: The individuals carrying the AC allele and the AG allele at the rs11624704 and the rs1004212 regions respectively had a high risk of being addicted to cigarettes.

Conclusion: This is first study investigating the relationship of the NRXN3 gene and nicotine addiction in the Turkish population. It was observed that the risk of NUD in the Turkish population may related to the Neurexin gene.

Keywords: Nicotine, gene, polymorphism, neurexin, addiction

INTRODUCTION

Substance Use Disorder (SUD) is a chronic disease characterized by the persistent loss of control over substance use despite demonstrating the negative outcomes of anxiety, dysphoria and other emotional, cognitive, and somatic symptoms resulting from compulsive substance-seeking behaviour, tolerance and withdrawal (Muskiewicz et al. 2018). Biological, behavioral and environmental factors play interacting roles in SUD. Excessive use of alcohol and nicotine has been reported as the main causes of preventable morbidity and mortality in western societies (Jha, 2009). Investigation of the causes of SUD should comprise all the risk factors including those underlying the genetic predisposition (Uluğ and Öztürk 2015). The common genetic background of the susceptibility to alcohol, nicotine or illegal substance use is highly heterogeneous and polygenic (Muskiewicz et al 2018, Jha and Peto 2014).

NUD not only lowers the quality of life but is also life threatening, in being associated with one out of five deaths in the world (Li et al. 2003) as only 10% to 20% of smokers do not develop NUD. (Perez Rubio and Cordopa-Lanus 2019). Whereas genetic factors are mainly causative in starting smoking, environmental factors are reported to be more dominant in the transition from smoking to use disorder (Sullivan and Kendler 1999, Bierut 2011, Akgün et al. 2011, Docampo et al. 2012, Şengül et al. 2016). Genetic transmision in the aetiology of NUD has been studied on families and twins accounting for 30-72% of the cases as well

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as molecular genetics based on the analysis of the functional candidate genes encoding proteins involved in known biological pathways in a particular disease ((Docampo et al. 2012, Perez Rubio and Cordopa-Lanus 2019). In NUD, the investigations were made on the genes encoding the nicotinic acetylcholine receptor, the genes that affect nicotine metabolism and the genes encoding the proteins associated with the noradrenaline, dopamine and serotonin system which, according to neurobiological studies, were thought to play a role in the effects of nicotine on the body and the pathophysiology of NUD (Bierut 2011). So far, the two classes of genes for the nicotine receptors, the CHRNA3-CHRNA5- CHRNB4 class located on chromosome 15 and the CHRNA6-CHRNB3 class located on chromosome 8 have been demonstrated to be the strongest genetic links to NUD (Docampo et al. 2012). The CHRNA5 gene shows several different allele frequencies between communities (Fang et al. 2017). Despite the reported strong association, the contribution of nicotinic receptor genes can only partly account for the susceptibility to NUD. Other genes may also contribute to NUD in an addictive or epistatic manner (Docampo et al. 2012).

The neurexin (NRX) gene family encodes a group of cell surface proteins or presynaptic cell adhesion molecules mainly expressed in neurons that mediate the conduction of nerve signals and affect the function of synapses (Perez Rubio and Cordoba-Lanus 2019). Cell adhesion molecules have been proposed to play a role not only in neural development but also in neuroplasticity that occurs throughout life (Moskiewicz et al. 2018). The NRX 1 and NRX 3 are two members of the NRX family associated with NUD (Şengül et al., 2016). The

The NRXN3 genes, transcribed from two promoters on 3 genes located on chromosome 14. show extensive alternative splicing result in more than 1000 isoforms distributed in different classes of neurons. Transcripts that initiate from upstream and down steam promoters encode, respectively, the α and β isoforms isoforms. The α-neurexins are alternatively spliced at five canonical positions, and the β-neurexins at two. NRXN3 is expressed in key circuits that play a role in SUD, including the glutamatergic neurons extending from the prefrontal cortex to the striatum and the GABAergic neurons. Therefore, NRXN3 is a strong candidate for SUD sensitivity (Docampo et al. 2012). NRXN3 has recently been associated with opioid, nicotine and alcohol use disorders (Moskiewicz et al. 2018, Wolock et al. 2013). Relationships were demonstrated between the NRX3 isoforms rs 8019381 and rs 760288 and alcohol use disorder (Hishimato et al. 2007) also confirmed in Turkey by Şengül et al. (2016); between rs 11624704 and attention deficit-impulsiveness and between rs 1004212 and alcohol problems (Stoltenberg et al. 2011). In schizophrenia, relationship was reported between the NRX 3 rs 1004212 polymorphism and ''smoking behaviour" (Novak et al. 2009) or ''heavy smoking" (Stoltenberg et al. 2011). Relationships were found between NRX3 rs221473 and NRX3 rs221497 and smoking behaviour in Spanish population and similar investigations in different populations were recommended (Docampo et al. 2012).

Relationships between NUD and the NRX3 gene polymorphisms of rs221473, rs221497, rs1004212 and rs11624704 demonstrated in different patient and population groups by others, have not been confirmed in the Turkish population In this study, it was aimed to verify the above described relationship of NRX3 gene polymorphisms with NUD and to analyse the effects of the genetic variants on the development and treatment of NUD in the Turkish population.

METHODS

Participants

The NUD group and the control group participants of this study consisted of 18-65 year old individuals. The NUD group included those without a psychiatric first axis disorder, mental retardation, past head trauma or a neurological disorder, who had smoked minimally 10 cigarettes per day for at least 1 year. The control group included individuals without any blood kinship with the NUD group, a serious chronic physical illness, a previous psychiatric disorder or mental retardation, who responded "no" to the question "have you ever smoked. It was deyermined by power analysis that the NUD group and the control group should minimally consist of, respectively, 123 and 132 individuals (Şengül et al. 2016). In a later period of the study, it was decided that each group should consist of 200 people against the possible loss of the questionnaire or DNA related data. The study was approved by Eskişehir Osmangazi University Faculty of Medicine Ethics Committee and was supported by Eskişehir Osmangazi University Scientific Research Project

The NUD and the control groups were recruited from volunteers working in the hospital and individuals living in their social environment who wanted to participate in the study. All volunteers were evaluated on the structured clinical interview (SCID-I) for DSM-IV Axis I Disorders and those who did not have Axis I diagnosis except for "Nicotine Addiction" were included in the NUD group of the study. The recruits were informed about the study and written informed consent on voluntary participation was obtained. Participants of the two groups completed a sociodemographic questionnaire and the NUD group also completed the Fageström nicotine dependence scale (FNDS). Subsequently, approximately 10 ml of venous blood samples of the participants were taken into tubes containing EDTA (ethylenediaminetetraacetic acid) for DNA extraction.

Data Acquisition Tools

The Structured Clinical Interview Scale for DSM-IV Axis-I Disorders (SCID-I): The SCID-I was developed by First et al.(1997) to investigate the diagnosis of axis I mental disorders according to DSM-IV. It was validated after adaptation to the Turkish language by Özkürkçügil et al. (1999).

The Fagerström Nicotine Dependence Test (FNDT): The FNDT was developed by Fagerström et al. (1992). The validity and reliability of the version in the Turkish language was conducted by Uysal et al. (2004) who reported the Cronbach's α coefficient as 0.56.

DNA Extraction and PCR Amplification

DNA was isolated from 5-10 µl of blood in accordance with the procedure outlined on the "Thermo Scientific GeneJET Genomic DNA Purification (USA)" kit. Hence, 20 µl proteinase K was placed at the bottom of a 1.5 ml eppendorf tube and 200 µl of the peripheral blood sample and 200 µl of extraction buffer was added; the contents were vortexed for 15 seconds, and thereafter kept in a water bath at 56°C for 10 minutes. The tubes were then centrifuged for 1 minute to remove the drops on the lid, followed by adding 200 µl binding buffer to the mixture, vortexing for 15 seconds and briefly centrifuging to remove the droplets on the lid. The mixture was transferred to the QIAamp Mini spin column without smearing the sides of the tube and centrifuged at 8000 rpm for 1 minute. The liquid at the bottom of the tube was poured out; 500 µl washbuffer II was added to the spin column and centrifuged at 8000 rpm for 1 minute. The liquid at the bottom of the tube was poured out and centriguged at 14000 rpm for 3 minutes. The spin column was placed in a clean 1.5ml Eppendorf tube ; 200 µl of elution buffer was added and centrifuged for 1 minute after incubating for 5 minutes at room temperature. The samples of DNA extract were stored at -20 ° C.

Neurexin gene polymorphisms rs 221473, rs 221497, rs1004212, rs11624704 were amplified by PCR. Reference sequence consisted of the Homo sapiens NRXN3 gene region. The PCR amplification mixture with a total volume 20 µl included 10 µl Master Mix TaqProb 2x (Abmgood, Canada), 1 µl probe (Applied Biosystems, Thermo Fisher Scientific, USA), 5 µl PCR grade d H2O and 4 µl DNA sample. PCR was performed by adjusting the Amplification Program Automatic heat cycling device (Step One Plus, Applied biosystems, Thermo Fisher Scientific, USA). The program included 1 cycle for 10 min at 95°C; 40 cycles over 3 sec at 95°C, 30 sec at 60°C and lastly 30 seconds at 25°C. After DNA amplification was completed, the temperature was raised very slowly to generate a melting curve for each sample. The gene polymorphisms were determined by distinguishing between the normal sequence and the sequence containing polymorphism during temperature raising.

Statistical Analysis

When all the questionnaire forms were evaluated at the end of the study, it was noticed that 5 people in the control group had answered questions directed to smokers indicating that they had a history of smoking for a period in the past. Therefore, the data of these 5 people were added to the smokers group, and statistical analyses were made on the data of 205 participants in the NUD group and of 195 participants in the control group.

In this study the IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, Illinois) and Medcalc 11.5.1 (OstendBelgium) programs were used for statistical analysis. The t-test was used to compare the data on age and years of education. Numerical data were expressed in terms by the mean ± the standard deviation (sd). Allele frequencies and genotype frequencies were analyzed by the Hardy-Weinberg equilibrium (HWE) and the chi-square test and were expressed in terms of frequency and percentage. Odds ratio was used for genotype comparisons, and logistic regression analysis was performed for significant genotypes and displayed as OR (95% Confidence Interval). The significance level was set at $p<0.05$.

RESULTS

The 205 participants of the NUD group consisted of 90 (43.9%) females and 115(56.1%) males, with 63(30.7%) being singles, 128 (62.4%) married/living together and 14 (6.8%) separated/widowed; 198 (96.6%) living in cities, 7 (3.4%)in villages/small towns; 133(64.9%) working regularly, 51(24.9%) unemployed, 5(2.4%) were working irregularly and 16 (7.8%) retired. The mean age of the NUD group was 37.31 ± 6.05 years with an average education period of 12.04 ± 4.44 years. The age at which they first tried smoking was 17.13 ± 3.92 years; the age they started to use regularly was 19.03 ± 3.96 years and the mean FNDS score was 4.67 ± 2.81. The 195 participants of the control group consisted of 129 (66.2%) females and 66 (33.8%) males, with 77 (39.5%) being singles, 109 (55.9%) married/living together and 9(4.6%) separated/widowed; 192 (99.5%) living in towns, 3 (1.5%) in villages/small towns; 143(73.3%) working regularly, 2 (1.0%) working irregularly and 2 (1.0%) retired. The mean age of the control group was 36.17 ± 6.21 years with an average education period of 12.57 ± 4.20 years. The two groups did not differ significantly with respect to age, education, marital status and place of residence ($p>0.05$). The percentage of females was higher in the control group in comparison to the NUD group. ($\chi^2=19.97$, $p<0.001$). Whereas the percentage of regular employement was higher in the control group, the percentage of retirement was higher in

Table 1. Demographic Data of the Participant GroupsComparison of the sociodemographic data of the groups

Variables	NUD (n=205) n (%)	Control-(n=195) n (%)	χ^2	P
Gender				
Female	90(43.90)	129(66.15)	19.97	<0.001
Male	115(56.10)	66(33.85)		
Marital status				
Single	63(30.73)	77(39.49)	3.76	0.150
Married/living together	128(62.43)	109(55.90		
Divorced/ widowed	14(6.84)	9(4.61)		
Residing in				
Village/small town	7(3.41)	3(1.53)	3.62	0.160
Major towns	198(96.69)	192(98.57)		
Working status				
Not working	51(24.87)	48(24.61)	12.39	<0.001
Works regularly	133(64.87)	143(73.33)		
Works irregularly	5(2.43)	2(1.03)		
Retired	16(7.80)	2(1.03)		
	mean ±ss	**mean ±ss**	**t**	**P**
Age	37.31 ± 6.05	36.17 ± 6.21	-1.31	0.190
Duration of education (years)	12.04 ± 4.44	12,57 ± 4.20	-1.22	0.220

the control group (χ^2=12.39, p <0.01). The sociodemographic data of the two groups are shown in Table 1.

Carrying the AA allele as compared to carrying the AC allele of the neurexin 3 gene rs11624704 was 2.33 times less associated with NUD (OR=0.43 95%CI 0.275, 0.672 p=0.0002). Whereas the carriers of the AC allele as compared to the carriers of the CC allele were 7.42 times more likely to develop NUD (OR=7.42 95%CI 2.80, 19.68 p=0.0001), the carriers of the AA alele as compared to the carriers of the CC gene were 3.19 times more likely to develop NUD (OR=3.19 95%CI 1.25, 8.14 p=0.0152). Hence, the probability of developing NUD appers to be higher for the carriers of the AC allele. Carrying the AA allele as compared to the AG allele of the neurexin 3 rs1004212 gene was 2.28 times less associated with NUD, but carrying the AG allele as compared to the GG allele was 2.27 times more associated with NUD. The likelyhood of developing NUD by the carriers of the AA and the GG alleles did not differ significantly. According to these results the AC allele was more associated with developing NUD.

Neurexin 3 rs221497 and rs221473 genes were not axxociated with NUD (p=0.667and p>0.05, respectively). The NRX3 rs11624704 and rs1004212 genotypes fit the Hardy-Weinberg equilibrium model (p=0.14 and p=0.31, respectively) with the posterior power analyses results being 99.61% and 95.19%, respectively. Comparison of the odds ratio of the genotyping in the NUD and control groups are shown in Table 2. Logistic regression analysis for the rs11624704 and rs1004212 genes with the variables of gender and employment status that differed significantly between the groups showed that the probability of developing NUD in males was 2.49 times higher than in females (p <0.001).

Table 2. Comparison of the Odds Ratios of the Genotypes in the NUD and the Control Groups

SNP	Genotypes	Odds Ratio (OR)	95% Cl	P
NRX3 rs11624704	AA vs AC	0.43	0.28-0.67	<0.001
	AC vs CC	7.42	2.80-19.68	<0.001
	AA vs CC	3.19	1.25-8.14	0.015
NRX3 rs1004212	AA vs AG	0.44	0.29-0.67	<0.001
	AG vs GG	2,27	1.01-5.12	0.050
	AA vs GG	1.01	0.45-2.23	0.989
NRX3 rs221497	CC vs CT	2.47	0.75-8.17	0.137
	CT vs TT	0.98	0.66-1.46	0.925
	CC vs TT	2.43	0.74-7.99	0.145
	AA vs AT	0.69	0.46-1.05	0.008
NRX3 rs221473	AT vs TT	4.08	0.79-20.72	0.093
	AA vs TT	2.81	0.56-14.18	0.211

Table 3. Results of Logistic Regression Analysis of Variables that Differ between Groups

Recessive model	n	OR(95%CI)	p value
AA+CC	272	1 (reference)	<0.001
AC	128	2.50 (1.61,3.88)	
AA+GG	234	1 (reference)	<0.001
AG	166	2.21(1.47,3.32)	
Female	219	1 (reference)	<0.001
Male	181	2.49(1.67,3.74)	

Table 4. Comparison of Groups according to Genotypes

Groups	NRX3 rs1162470 genotypes						χ^2; P
	AA		AC		CC		
	n	%	n	%	n	%	
NUD (n=205)	114	55.6	85	41.5	6	2.9	24.64; <0.001
Control (n=195)	131	67.2	42	21.5	22	11.3	
	NRX3 rs1004212 genotypes						
	AA		AG		GG		
	n	%	n	%	n	%	
NUD (n=205)	89	43.4	104	50.7	12	5.9	15.60; <0.001
Control (n=195)	118	60.5	61	31.3	16	8.2	
	NRX3 rs221497 genotypes						
	CC		CT		TT		
	n	%	n	%	n	%	
NUD (n=205)	10	4.9	93	45.4	102	49.8	2.37; 0.667
Control (n=195)	4	28.6	92	47.2	99	50.8	
	NRX3 rs221473 genotypes						
	AA		AT		TT		
	n	%	n	%	n	%	
NUD (n=205)	118	57.6	85	41.5	2	1.0	5.28; 0.259
Control (n=195)	126	64.6	63	32.3	6	3.1	

Table 5. The Allelic Frequencies of Single Gene Polymorphisms of the NRX3 Gene in the Participant Groups

SNP-Alel	NUD (N=205) n(%)	Control (N=195) n(%)	OR(%95 CI)	P values
rs11624704				
A	313(76.34)	304(77.94)	1.079 (0.776-1.501)	0.650
C	97(23.66)	86(22.06)		
rs1004212				
A	282(68.78)	297(76.15)	1.066 (0.793-1.434)	0.672
G	128(31.22)	93(23.85)		
rs221497				
C	113(27.56)	100(25.64)	1.186 (0.868-1.620)	0.284
T	297(72.44)	290(74.36)		
rs221473				
A	321(78.29)	315(80.77)	1.165 (0.825-1.643)	0.386
T	89(21.71)	75(19.23)		

Employment status factor did not affect the probability of developing NUD.

In NRX3 rs1162470 genotypes, carrying the AC allele as compared to the AA + CC alleles increases the probability of the developing NUD by 2.50 fold (p <0.001). Also, in NRX3 rs1004212 genotypes; the probability of developing NUD is 2.21 times higher in carriers of the AG allele as compared to the carriers of the AA + GG alleles (p <0.001). The logistic regression analysis results of the variables with differences between the groups are shown in Table 3. Comparison, of the results between smoking status based on genotypes are shown in Table 4, and allele frequencies of single nucleotide polymorphisms investigated for NRX3 are shown in Table 5.

DISCUSSION

According to the results of this study, the neurexin 3 gene alleles AC for rs11624704 and AG for rs1004212 are associated with NUD. Stoltenberg et al. (2011) found a moderate correlation between the NRXN3 rs11624704 polymorphism and impulsiveness in males but not in females. A 2.54 fold higher risk of alcohol use disorder was observed in male carriers of the NRXN3 rs1004212 T allele as compared to the carriers of the CC allele. Having observed a low risk of SUD in female cariers of the NRXN3 rs1004212 T allele, it was argued that the correlation between NRXN3and SUD and any gender based differences would become clearer

with accumulated evidence. In schizophrenia patients the NRXN3 rs1004212 C/C genotype correlated more with NUD than the C/T genotype (Novak et al.2009). However, neither of these two studies identified NRXN3 rs1004212 polymorphism with the risk of NUD.

The NRX3 rs760288 polymorphism (Şengül et al. 2016) and the rs 8019381 polymorphism (Hishimoto et al. 2007) were reported to be associated with alcohol use disorder. In our study, we did not evaluate NRXN3 gene polymorphism in relation to impulsivebess. Therefore, although our results support the relationship between NUD and the NRXN3 rs 11624704 and rs 1004212 polymorphisms, data on gender based variation of this relationship is not provided. The numbers of male participants of the NUD group were significantly higher in our study. The 2019 data of the Turkey Statistics Institute show 41.3% of men and 14.9% of women as smokers. The results of logistic regression analysis in this study indicated 2.49 times higher probability of developing NUD among the male as compared to the female participants. The significant difference in the employment status of the NUD and control groups of our study was not associated with the risk of NUD. While Stoltenberg et al. (2011) worked with participants using different substances, we only worked with NUD in our study. Also, whereas Novak et al. (2009) worked with schizophrenia patients, Axis I disorders, except for NUD, were excluded in our study. Therefore, our results are specific to the relationship between NRXN3 gene polymorphism and NUD. Observation of a low risk relationship between NUD and the NRX3 rs221473 and NRX3 rs 221497genotypes in the Spanish population prompted the suggestion for repeating these investigations with larger groups of participants in different populations (Docampo et al. 2012). In our study, however, correlations between NUD and the NRX3single-nucleotide polymorphism regions rs 221473 and NRX3 rs 221497 were not demonstrable. Recently, 3 studies investigating the relationship between nicotine addiction and single nucleotide gene polymorphism were published in our country. Yukseloğlu et al. (2019), examining buccal swab samples in the Anatolian population for 10 candidate gene single nucleotide polymorphisms (SNP), could not demonstrate a significant correlation with NUD and recommended that the variations detected in the single nucleotide polymorphism loci rs16969968, rs806380, rs3733829**,** rs6474412, rs1329650 should be repeated with larger participant groups. Müderrisoğlu (2019) reported moderate and mild correlations between NUD and the CHRNA4 rs1044396 AA (p=0.02), the CHRNA5 rs16969968 A (p=0.003), the MDR1 rs1128503 T (p=0.01), the MDR1 rs2032582 T/A (p=0,018 genotypes and the MDR1 TT-TT-TT haplotypes (p= 0.047). Also, the CHRNA3 rs578776 T allele was associated with low incidence in giving up smoking after nicotine replacement therapy (NRT) combined with bupropion treatment; while the nNOS exon 29 TT genotype was associated with a higher incidence of smoking cessation with bupropion treatment. It was proposed that the CHRNA3 rs578776, CHRNA4 rs1044396, CHRNA5 rs16969968, MDR1 rs1128503, MDR1 rs2032582 and the nNOSekzon 29 polymophisms may contribute as new biomarkers in the pharmacogenomic optimization of smoking addiction treatment. Uysal et al. (2019) found a significant difference between smoking and non-smoking groups in relation to the DRD4 gene and the allele distribution. Accepting 48 base pair repeat alleles as six repeats or shorter (K) and 7 repeats or longer (U), they claimed that the DRD4 VNTR exon 3 K/U genotype was associated with 2.25 times higher risk of NUD. It is seen that the few studies reported in the literature in our country were conducted on different genes. Given the multiplicity of biological, psychological and social factors involved in nicotine addiction, our study has concentrated on the effect of the NRXN3 single gene polymorphisms on genetic predisposition to NUD.

Our study is the first to investigate in a sample of Turkish population, nicotine addiction in relation to the NRXN3gene on its regions previously associated with addictive behaviours in different investigations. Our results point to a significantly high risk relationship between NUD and the Neurexin 3 gene rs11624704 and rs1004212 single gene polymorphisms. Although heritability of addiction has been strongly demonstrated by candidate gene studies conducted outside our country, the failure to replicate the results on linkage and association with candidate gene loci is due to the heterogeneity of addiction genetics (Moskiewicz et al., 2018). Therefore, further investigation on the results of these studies with larger population groups will be helpful in understanding the genetic predisposition to nicotine abuse in Turkey.

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