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STUDY OF NEUROPILIN-2 RS849563 GENE POLYMORPHISM IN CHILDREN WITH AUTISM SPECTRUM DISORDER

Zeynab MAMMADOVA[1], Ikram RUSTAMOV[2], Narinj RUSTAMOVA[3], Gular IBRAHIMOVA[1], Mehraj ABBASOV[1, 4], Sevda BABAYEVA[1*]

[1]Genetic Resources Institute, Ministry of Science and Education of the Republic of Azerbaijan, AZ1106, Baku, Azerbaijan
[2] Nefes psychoneurological center, Baku, Azerbaijan
[3] Narinj Psychology Center, Baku, Azerbaijan
[4]Western Caspian University, AZE1072, Baku Azerbaijan

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Autism Spectrum Disorder (ASD) is a significant developmental condition in children, resulting from a combination of genetic and environmental factors. This study focused on analyzing the NRP2 (rs849563) gene variation and its association to autism risk in a group of 36 Azerbaijani children, with 18 having ASD and 18 as controls. The genotyping of the single nucleotide polymorphism (SNP) was carried out using PCR-RFLP analyses. The results of the analysis showed that in autistic children, 61.1% had the TT genotype, and 38.9% had the TG genotype, while in the control group, the frequencies were 55.6% for TT and 44.4% for TG. The GG genotype was not found in either group. Statistical analysis revealed no clear link between genotypes and the likelihood of developing autism (OR = 0.7955, 95% CI = 0.21 - 3.00, P = 0.7355). The T and G allele frequencies were 80.6% and 19.4% in the ASD group, and 77.8% and 22.2% in the control group, respectively. The study suggested that the NRP2 (rs849563) T allele might be associated with a higher risk of autism compared to the G allele, according to binary logistic regression analysis (OR = 1.18, 95% CI = 0.38 to 3.7). However, this association

Corresponding author: Sevda Babayeva, Genetic Resources Institute of Ministry of Science and Education of Azerbaijan, Azadlig Ave 155, AZ1106, Baku, Azerbaijan, e-mail: seva_genetic@yahoo.com, phone: +994505327819, ORCID: 0000-0002-0888-4539, Z. Mammadova ORCID: 0009-0005-6812-8005, I. Rustamov ORCID: 0009-0008-5144-840X, Z. Mammadova ORCID: 0009-0005-6812-8005, N. Rustamova ORCID: 0000-0003-3514-7937, G. Ibrahimova ORCID: 0000-0001-7631-7221, M. Abbasov ORCID: 0000-0002-2564-3411

did not reach statistical significance (P = 0.7718). Additionally, when comparing the genotypes of parents and autistic children, a transmission disequilibrium analysis showed no significant imbalance for the rs849563 marker (LRS=1.14, df=1, P=0.29).

Keywords: ASD, gene, polymorphism, *NRP2*, PCR-RFLP

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurological and complex multifactorial developmental condition that becomes evident within the first three years of life. It is characterized by challenges in social relationships, communication, speech development, as well as repetitive behaviors and restricted interests (LEBLANC and GILLIS, 2012). Epidemiological studies indicate a continuous rise in the number of individuals diagnosed with autism each year. The estimated prevalence of the disorder is around 0.2-0.5%. Notably, the risk of autism is approximately four times higher in boys compared to girls, with a sex ratio of about 4.2 to 1 (GESCHWIND *et al*., 2007).

Epidemiological studies have identified various risk factors associated with autism, yet it remains unclear which of these factors is essential or adequate for the onset of autism (RODIER, 2011). Research suggests that abnormal cell migration, synaptic pruning, and programmed cell death might play a role in the etiology of autism. These mentioned characteristics are influenced by numerous factors during early stages of development, particularly genetic, hormonal, and biochemical factors (YORBIK *et al*., 2002).

Family and twin studies have substantiated that hereditary factors contribute significantly, accounting for up to 50% of involvement in autism (HALLMAYER *et al*., 2011; SANDIN *et al*., 2017). While chromosomal abnormalities or single gene mutations have been considered as potential causes of the disease, no specific candidate genes for Autism Spectrum Disorder (ASD) have been conclusively identified. Moreover, recognized mutations and copy number variants (CNVs) only account for approximately 10-20% of ASD cases, emphasizing the necessity for extensive genetic investigations to identify undiscovered genetic factors. The genesis of ASD might be influenced by alterations in crucial genes responsible for normal brain development, growth, cognitive functions, and behavior (LIU *et al*., 2011).

It has been established that neuropilin genes (NRPs), responsible for encoding proteins crucial to neurodevelopment, might play a role in the pathogenesis of autism. NRPs are transmembrane glycoproteins specific to vertebrates, featuring two homologues: neuropilin-1 (NRP1) and neuropilin-2 (NRP2). The human NRP1 and NRP2 genes encode complete proteins, each comprising 923–926 amino acids. Positioned on chromosomes 10p12 and 2q34, respectively (ROSSIGNOL *et al*., 1999), the NRP1 gene spans 120 kb, while the NRP2 gene exceeds 112 kb, both encompassing 17 exons (ROSSIGNOL *et al*., 2000). The structural similarities observed in the sizes of exon and intron regions, the exon-intron arrangement, and the positions of splicing sites suggest that these genes originated through duplication. Neuropilins serve as co-receptors, exhibiting a robust affinity for binding extracellular ligands, and they collaborate with other transmembrane receptors to form holo-receptors (PELLET-MANY *et al*., 2008). Within the central nervous system, NRP2 is essential for directing axons and regulating the migration of neurons, as highlighted by GIGER *et al*. (2000). Multiple research groups have investigated the impact and mechanisms associated with the NRP2 gene on neuronal

proliferation, apoptosis, and migration (CHEN *et al*., 2000). Nonetheless, the exploration of the connection between NRP2 and autism has been relatively limited, with only a few studies addressing this relationship (HOSSEINPOUR *et al*., 2017).

Until now, the predominant focus of genetic association studies on autism spectrum disorders has been within North America, Western Europe, and Australia (SUN *et al*., 2015). Genetic research on autism in the South Caucasus region is infrequent, although studies have been undertaken in Russia and the Baltic post-Soviet republics (GIBITOVA *et al*., 2022). As per the 2020 report from the Mental Health Centre of the Ministry of Health, our country has 1383 registered autistic children, with 46 of them being classified as atypical (https://report.az/sehiyye-xeberler/azerbaycanda-autizm-sindromu-olan-usaglarin-sayi-aciqlanib/).

Psychopedagogical methods, including speech therapy and organized training, are employed for the identification and treatment of autism in these children. In a recent study by Rustamova, focusing on 1473 children with Autism Spectrum Disorder, an autistic spectrum index for each child was estimated using the Gilliam ASD Rating Scale (RUSTAMOVA, 2023). To formulate a comprehensive strategy for addressing our country's autism challenges, it is necessary to conduct genetic research aimed at identifying the genetic markers responsible for Autism Spectrum Disorder.

Hence, the primary objective of the present study was to investigate the NRP2 (rs849563) gene polymorphism within the Azerbaijani population and determine whether there exists an association between this polymorphism and the risk of developing autism.

MATERIAL AND METHODS

The current study involved a total of 18 patients diagnosed with autism spectrum disorder, who received treatment at the "Nefes" psychoneurological center, along with 18 control participants. The control group comprised healthy children with no family history of psychological diseases. Both patients and controls were selected from the same population. The diagnosis of Autism Spectrum Disorder was made using the DSM-5 criteria. Individuals with a previous diagnosis of chromosomal abnormalities, dysmorphic characteristics, tuberous sclerosis, fragile X syndrome, and any other neurological disorder associated with autism were excluded from the study. Buccal (cheek) samples were collected from both the children with ASD and the control group to extract genomic DNA. Informed permission was obtained from the parents of autistic patients for their participation in the study.

Genomic DNA extraction

DNA extraction was performed at the Department of Molecular Genetics and Genomics, Laboratory of Human Genetics within the Genetic Resources Institute (GHATAK *et al*., 2013). Subsequently, the quantitative and qualitative characteristics of the extracted DNA were assessed using nanodrop equipment (THERMO SCIENTIFIC, 2000).

Analysis of genetic polymorphism

In this study, genotyping of the NRP2 gene polymorphism (rs849563) was conducted through PCR-RFLP. PCR reactions were performed using the following primers for the

rs849563 polymorphism of the NRP2 gene: 5′- TGACCAGGAATCAA CTAGGAAGC-3′ (forward) and 5′- CGACCACCTCTCCG GGTAT-3′ (reverse). The amplification solution was 20 µl in volume and had the following composition: 2 µl DNA, 2 µl 10X buffer [10 mMTris–HCl pH 8.0, 50 mM KCl], 2 µl $MgCl_2$, 0.2 µl 10mM dNTP mixture, 0.5 µl 100 µM primer and 0.2 µl 5 U/ µl Taq polymerase enzyme. The PCR process comprised an initial denaturation stage (5 minutes at 95 °C), followed by 35 cycles (30 seconds at 95 °C, 1 minute at 60 °C, 2 minutes at 72 °C), and a final elongation stage (5 minutes at 72 °C). Subsequently, PCR amplicons underwent processing through the PCR-RFLP method using the BSAJI restriction enzyme (New England Biolabs) with an incubation temperature of 60 °C, and the outcomes were analyzed on a 3% agarose-ethidium bromide gel under UV light.

The obtained results were subjected to statistical evaluation, and allele and genotype frequencies were determined for both groups. This methodology allows for the identification of all three possible genotypes for the polymorphism: homozygous wild type, heterozygous variant type, and homozygous variant type.

Statistical analysis

Statistical analyses were performed using MedCalc version 12.1. The genotype frequencies of the NRP2 polymorphism in both the autistic and control groups were assessed using the $\chi 2$ test. Binary logistic regression analysis was employed to determine odds ratios (OR) and 95% confidence intervals (CI) for the genetic polymorphisms. Additionally, a transmission disequilibrium test was executed for alleles. Results were considered statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

As mentioned earlier, Autism Spectrum Disorder (ASD) is a neurodevelopmental condition. The manifestation of motor impairments in autistic children, noticeable in the initial months of life and sometimes even at birth, provides further backing to the neurodevelopmental interpretation of autism (TEITELBAUM *et al*., 1998). Various mutations in neuropilin genes have been identified, contributing to the pathogenesis of certain disorders. Studies have revealed that single nucleotide polymorphisms within the 2q34 region of the human genome, responsible for coding neuropilin 2, are linked to autism (WU *et al*., 2007).

The current study represents a genetic analysis of autism for the first time in Azerbaijan, incorporating an association analysis for the SNP marker rs849563 located in the 10th exon of the Neuropilin-2 gene. In our investigation, 77.7% of individuals diagnosed with autism were males, while 22.2% were females, in contrast to 55.5% and 44.4% in the control group, respectively. The average age of the patients was 5.11 ± 1.80 (range: 2–17), and the control group had an average age of 6.12 ± 1.62 (range: 2–15). Behavioral parameters, based on deviations from the typical behavior model, were assessed using the clinical records of autistic children and interviews conducted with the participation of a neurologist. Among the 18 children diagnosed with Autism Spectrum Disorder, 12 (66.6%) experienced speech delay, 8 (44.4%) exhibited hyperactivity, and 5 (27.7%) displayed additional clinical signs like developmental delay. Clinical data for three children were not available (Table 1).

The χ^2 test was performed to identify a polymorphism between children with ASD and control groups. The results of the analysis found no significant difference ($P \geq 0.05$) in the allele and genotype frequencies of rs849563 in the control and ASD groups.

Table 1. Demographic and clinical characteristics of patients and controls

Characteristic	ASD group	Control
	Gender	
Male	14 (77,7%)	10 (55,5%)
Female	4 (22,2%)	8 (44,4%)
	Age	
Age ranges	2-17	2-15
Average age	5.11 ±1.80	6.12 ±1.62
	Clinical signs	
Speech delay	12 (66,6%)	
Hyperactivity	8 (44,4%)	
Developmental delay	5 (27,7%)	

Upon cutting DNA fragments with the BSAJI restriction enzyme, the following observations were made: 230 bp for the T/T genotype, 230 bp, 163 bp, and 67 bp for the T/G genotype, and 163 bp and 67 bp for the G/G genotype (Figure).

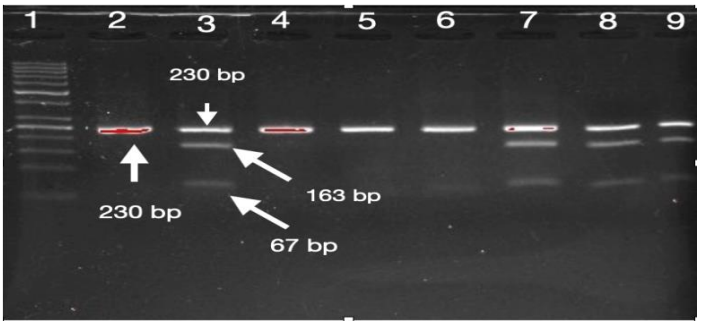

Fig. Agarose-gel electrophoresis of NRP2 rs849563 polymorphism**.** 1-Ladder (50 bp), 2,4,5,6 - TT genotype, 3,7,8,9 - heterozygous GT genotype

Allele and genotype frequencies observed in children with ASD and control group are presented in Table 2.

Analysis of the results revealed that the prevalence of TT and TG genotype frequencies in autistic children was 61.1% and 38.9%, respectively, while in the control group, it was 55.6% and 44.4%. Notably, the GG genotype was absent in both groups, aligning with findings in the Iranian population where the GG genotype was not detected in any of the 120 case-control samples (HOSSEINPOUR *et al*., 2017). In our study, a statistical analysis indicated no discernible

association between genotypes and the risk of autism (OR = 0.7955, 95% CI = 0.21–3.00, P = 0.7355).

Table 2. Allele and genotype frequencies observed in children with ASD and controls

	Autism (n=18)	Control (n=18)	OR(95%CI)	P
Alleles (T → G)				
T	14.5 (80.56%)	14 (77.78%)	1.00 (reference)	-
G	3.5 (19.44%)	4 (22.22%)	0.84 (0.27 - 2.64)	0.7718
Genotypes				
TT	11 (61.11%)	10 (55.56%)	1.00 (reference)	-
TG	7 (38.89%)	8 (44.44%)	0.79 (0.21 - 3.00)	0.7355
GG	0 (0)	0 (0)	0	-

The frequency of occurrence of T and G alleles in children with autism was 80.6% and 19.4%, respectively, while in the control group, these figures were 77.8% and 22.2%. Binary logistic regression analysis indicated that although the T allele of the NRP2 (rs849563) gene was associated with a higher risk of autism compared to the G allele (OR = 1.18, 95% CI = 0.38–3.7), the difference did not reach statistical significance (P = 0.7718).

In addition to analyzing the ASD and control groups, we conducted a genotyping comparison between children with autism and their parents, along with an examination of the rs849563 marker alleles based on transmission disequilibrium. The transmission disequilibrium test serves as a family-based association test to establish a connection between a genetic marker and a trait. This method evaluates the excess or over-transmission of an allele from heterozygous parents to offspring with any pathology. The results revealed that the rs849563 marker did not display transmission inequality (LRS = 1.14, df = 1, P = 0.29) (Table 3).

Table 3. Transmission disequilibrium test for allelic association between Single Nucleotide Polymorphism and autism

Marker	Allele	T [a]	NT [b]	T-frequency [c]	LRS [d]	P
rs849563	T	9	5	0.64	1.14	0.29
	G	5	9	0.36		

Note: a and b - number of transmission and non-transmission of each allele, respectively; c – transmission frequency; d - likelihood ratio statistic

In another study, WU *et al*. (2007) explored the association between the NRP2 gene and autism using a cohort of 169 Chinese families (children with autism and their parents). The findings disclosed a significant genetic association between autism and two SNPs of the NRP2 gene (rs849578, rs849563), as well as specific haplotypes, particularly those formed by the rs849563 SNP.

Following the investigation of the rs849563 polymorphism of the Neuropilin-2 gene within the Azerbaijani population, which included 36 samples in the case-control group, no statistically significant dependence was identified between the groups concerning genotype and allele frequency. The variations in results observed for the NRP2 rs849563 polymorphism across different populations could be attributed to differences in sample size and the influence of other genetic and environmental factors. In our study, the limited number of samples stands out as a primary constraint. Consequently, additional research is essential to validate the involvement of the NRP2 gene in Autism Spectrum Disorder.

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ISTRAŽIVANJE POLIMORFIZMA NEUROPILIN-2 RS849563 GENA KOD DECE SA POREMEĆAJEM AUTISTIČKOG SPEKTRA

Zeynab MAMMADOVA[1], Ikram RUSTAMOV[2], Narinj RUSTAMOVA[3], Gular IBRAHIMOVA[1], Mehraj ABBASOV[1, 4], Sevda BABAYEVA[1*]

1 Institut za genetičke resurse, Ministarstvo nauke i obrazovanja Republike Azerbejdžan, Baku, Azerbejdžan
2 Nefes psihoneurološki centar, Baku, Azerbejdžan
3 Psihološki centar Narinj, Baku, Azerbejdžan
4 Vestern Caspian Univerzitet, Baku, Azerbejdžan

Izvod

Poremećaj autističnog spektra (ASD) je značajno razvojno stanje kod dece, koje je rezultat kombinacije genetskih i faktora sredine. Ova studija se fokusirala na analizu varijacije gena NRP2 (rs849563) i njene povezanosti sa rizikom od autizma u grupi od 36 azerbejdžanske dece, od kojih je 18 imalo ASD i 18 kao kontrole. Genotipizacija polimorfizma jednog nukleotida (SNP) je sprovedena korišćenjem PCR-RFLP analiza. Rezultati analize su pokazali da je kod autistične dece 61,1% imalo TT genotip, a 38,9% TG genotip, dok je u kontrolnoj grupi učestalost bila 55,6% za TT i 44,4% za TG. GG genotip nije pronađen ni u jednoj grupi. Statistička analiza nije otkrila jasnu vezu između genotipova i verovatnoće razvoja autizma (OR = 0,7955, 95% CI = 0,21 - 3,00, P = 0,7355). Učestalost alela T i G iznosila je 80,6% i 19,4% u ASD grupi, a 77,8% i 22,2% u kontrolnoj grupi. Studija je sugerisala da bi alel NRP2 (rs849563) T mogao biti povezan sa većim rizikom od autizma u poređenju sa alelom G, prema analizi binarne logističke regresije (OR = 1,18, 95% CI = 0,38 do 3,7). Međutim, ova povezanost nije dostigla statistički značaj (P = 0,7718). Pored toga, kada se uporede genotipovi roditelja i autistične dece, analiza transmisione neravnoteže nije pokazala značajnu neravnotežu za marker rs849563 (LRS=1,14, df=1, P=0,29).

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