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Association of NRP2 rs849563 polymorphism with autism spectrum disorder and related clinical variables in Azerbaijani children

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Abbreviations
ASD – autism spectrum disorder
DSM – diagnostic and statistical manual
GARS – Gilliam Autism Rating Scale
NRP – neuropilins

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Abstract

Background and purpose: *Autism, a prevalent developmental disorder, has been linked to genetic factors that regulate critical brain functions, such as neuropilins (NRPs). This study aimed to examine the NRP2 rs849563 polymorphism for its association with autism risk and its relationship with specific autism spectrum disorder (ASD) clinical variables in the Azerbaijani population.*

Materials and methods: *The study included 48 ASD patients and 42 controls from the same population. Diagnoses followed DSM-5 criteria, with DNA extracted from buccal samples and genotyped for the NRP2 rs849563 polymorphism via PCR-RFLP. Statistical analyses using MedCalc assessed genotype frequencies and their associations with clinical variables, with significance set at* $p \leq 0.05$.

Results: *PCR-RFLP genotyping revealed 75% TT and 25% TG in autistic children, compared to 66.67% TT and 33.33% TG in controls, with the GG genotype absent. Statistical analysis showed no significant association of genotype (OR = 0.667, 95% CI = 0.267 - 1.666,* $p = 0.385$*) or allele frequencies (OR = 0.714, 95% CI = 0.31 – 1.64, p = 0.429) with ASD. However, a significant association was found between the NRP2 rs849563 polymorphism and rocking and swaying behavior (*$p = 0.029$*), highlighting its potential role in ASD symptomatology.*

Conclusions: *Our study provides pioneering insights into the potential role of the NRP2 gene in autism susceptibility among Azerbaijani children. The results, including a significant association, lay the groundwork for future research with larger cohorts and more comprehensive phenotypic evaluations.*

INTRODUCTION

Autism Spectrum Disorder (ASD) is a complex neurological condition manifesting within the first three years of life. Autistic individuals exhibit unique cognitive deficiencies, such as impaired social cognition, executive dysfunction, and unusual perception and information processing. These characteristics are supported by abnormal neurodevelopment at the system level (*1*). It has been claimed that cerebral cortical anomalies observed in neuroimaging studies of autistic people may stem from neuronal migration disorders that disrupt the proper positioning of neurons within the cerebral cortex (*2*).

ASD affects around one out of every hundred children worldwide. Prevalence estimates increased over time and varied significantly inside

and across sociodemographic groups (*3*). Autism is almost four times more common in boys than in girls. Although the etiology of autism is uncertain, family and twin studies have shown an important role of genetic variables in the development of autism. Specific ASD candidate genes remain unidentified, with known mutations explaining only 10-20% of cases, prompting further genetic research (*4*). Genome-wide association studies have uncovered some significant single nucleotide polymorphisms, but none have a large enough influence to be considered causal. However, up to 40% of simplex families and 60% of multiplex families (those in which more than one person has autism) may contain numerous single nucleotide polymorphisms that, when combined, have an additive influence on autism risk (*5*).

Neuropilin genes (NRPs) are implicated in autism pathogenesis due to their role in neurodevelopment. The neuropilin receptors and their secreted semaphorin ligands play vital roles in brain circuit development by regulating several essential neuronal processes, including synaptic maturation and migration of GABAergic interneurons (*6*).

NRP2, a transmembrane glycoprotein consisting of 926 amino acids, is encoded by a gene on chromosome 2q34 (*7*). Animal model studies have shown that NRP2 is crucial for the organization and fasciculation of cranial and spinal nerves (*8*). Beyond axon guidance, NRP2 plays a key role in regulating neuronal migration within the peripheral and central nervous systems, making it a candidate gene for autism (*6*). Wu *et al.* found a significant genetic association between autism and multiple polymorphisms in the NRP2 gene, including rs849563 located in exon 10, with a particular emphasis on haplotypes involving this specific SNP. Single-locus testing using TDTPHASE revealed nominally significant transmission disequilibrium for rs849563, supporting its potential involvement in autism (*9*). Furthermore, a significant association between the rs849563 polymorphism and autism has been reported in the Iranian population (*10*).

Based on findings from previous studies, the objective of the current work was 1) to explore the NRP2 rs849563 polymorphism in the Azerbaijani population and assess its potential association with autism risk, and 2) to evaluate the relationship between specific ASD clinical variables and the selected SNP. This represents among the first genetic data on autism in the Azerbaijani population.

MATERIAL AND METHODS

The study included 48 patients with autism spectrum disorder treated at the "Nefes" psychoneurological center and Narinj psychology center, all diagnosed with ASD according to DSM-5 criteria. Patients were evaluated by an expert psychiatrist and a neurologist specialized in child and adolescent health. Only individuals with no personal or family history of psychiatric disorders, such as Alzheimer's disease, Parkinson's disease, or schizophrenia, and no previously identified chromosomal abnormalities were included. The control group consisted of 42 subjects from the same population, with no personal or first-degree family history of mental health or psychiatric disturbances, as determined by personal interviews.

The Gilliam Autism Rating Scale (GARS-3) was used for a comprehensive evaluation of traits across 30 children. GARS-3 has six subscales: Restrictive/Repetitive Behaviors, Social Interaction, Social Communication, Emotional Responses, Cognitive Style, and Maladaptive Speech. Scores on the GARS range from 0 to 3 for each item: 0 indicates that the item does not describe the individual's behavior at all, while 3 indicates that the item very much describes the individual's behavior. A cumulative GARS score above 71 indicates that autism spectrum disorder is very likely (*11*). Additionally, several ASD-related traits, including eye contact, repetitive behaviors, speech, cognitive understanding, echolalia, rocking and swaying, response to name, and hyperactivity were recorded through observations by experienced psychologists at the psychology centers for all 48 children.

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Genetic Resources Institute, Ministry of Science and Education. Buccal samples were used for DNA extraction (*12*), and informed consent was obtained from the parents or guardians of both autistic patients and control subjects prior to their participation in the study.

The NRP2 rs849563 polymorphism in the 10th exon was genotyped using PCR-RFLP. PCR reactions with specific primers and digestion with the BSAJI (Thermo Scientific Eco47I) restriction enzyme were conducted according to the protocol described by Hosseinpour *et al.* (*10*). Upon BSAJI restriction enzyme cutting, T/T genotype showed 230 bp fragment, T/G showed 230 bp, 163 bp, and 67 bp, and G/G showed 163 bp and 67 bp.

Statistical analyses were performed using MedCalc version 12.1 (*13*). Genotype frequencies of the NRP2 polymorphism in both the autistic and control groups were assessed using the χ^2 test. Binary logistic regression analysis was used to determine odds ratios (OR) and 95% confidence intervals (CI) for genetic polymorphisms. Additionally, a χ^2 test was conducted to examine any association between seven ASD related clinical variables (Table 2) and SNP alleles. Results were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Neuropilins, such as NRP2, guide axons and regulate neuronal migration, with studies in null mice displaying behaviors similar to autism (*6*). Several studies highlight the significant association between rs849563 polymorphism of the NRP2 gene and ASD risk (*9, 10*).

Table 1. *Allele and genotype frequencies observed in children with ASD and controls in Azerbaijani population.*

	Autism (n=48)	Control (n=42)	Odds ratios (95%CI)	*p*-Value
Alleles				
T	84 (87.50%)	70 (83.33%)	0.71 (0.31–1.64)	0.429
G	12 (12.50%)	14 (16.67%)		
Genotypes				
TT	36 (75%)	28 (66.67%)	0.67 (0.27–1.66)	0.385
TG	12 (25%)	14 (33.33%)		

Following this, the current study represents among the earliest genetic analyses of autism in Azerbaijan, focusing on the rs849563 polymorphism of the NRP2 gene in a sample of 90 individuals. In this study, 72.92% of individuals diagnosed with autism were males, while 27.08% were females, compared to 54.76% male and 45.24% female in the control group. The median age of patients was 4.05 years (range: 1.8–17), while the median age of the controls was 5.0 years (range: 1.5–18), with statistical analysis indicating significant differences in age distribution between the groups (p-value ≤ 0.05). Among 48 autistic children, only 3 had a positive family history. Hyperactivity was observed in 28 children, with 5 classified as having moderate hyperactivity and 2 as having severe hyperactivity. GARS scores were recorded for 30 children in the ASD group, with scores ranging between 71 and 110. The mean GARS score was 89.90 ± 3.55. Among the 48 autistic children, only 2 had a GARS score of 101 or higher, indicating a need for very substantial support (Level 3). The remaining children were classified in Level 2, which denotes a requirement for substantial support.

Table 2. *Association of clinical phenotype variables of ASD with NRP2 rs849563 alleles.*

Clinical Phenotype	Classification	NRP2 rs849563 alleles			
		T	G	*p*-Value	χ^2
Eye contact	Good	22	0		
	Poor	22	4	0.17	3.5
	No	28	4		
Repetitive behaviors	Yes	50	6	0.81	0.06
	No	11	1		
Rocking and swaying	Yes	13	3	**0.029**	4.75
	No	41	1		
Speech	No speech	28	2	0.32	2.26
	Only a few single words	24	2		
	Able to make short sentences	23	5		
Understanding (Cognitive)	Good	11	1	0.70	0.72
	Limited	25	1		
	Poor	18	2		
Response to name	Yes	28	2	0.24	2.83
	Poor	10	0		
	No	32	6		
Echolalia	Yes	26	4	0.39	0.73
	No	28	2		

The χ^2 test revealed no significant differences between ASD and control groups (p-value > 0.05). Analysis of the results revealed that the frequencies of TT and TG genotypes in autistic children was 75.0% and 25.0%, respectively, while in the control group, it was 66.7% and 33.3%. Notably, the GG genotype was absent in both groups (Table 1). Statistical analysis indicated no discernible association between genotypes and the risk of autism (OR = 0.667, 95% CI = 0.267-1.666, p-value 0.385).

T and G allele frequencies were 87.5% and 12.5% in ASD children, and 83.3% and 16.7% in controls, with statistical analysis indicating no significant association between frequencies of NRP2 rs849563 alleles and ASD risk (OR = 0.714, 95% CI = 0.31 – 1.64, p=0.429).

In contrast, a study conducted in a Chinese population identified a significant genetic association between autism risk and the rs849578 and rs849563 polymorphisms in 169 families (*9*). In another study, a 2.3-fold increased disease risk was reported with the NRP2 G allele compared to the T allele in an Iranian population comprising 50 ASD and 70 control subjects. In addition, the study found that heterozygous TG was associated with a higher incidence of autism than the TT genotype (*10*). This disparity in NRP2 rs849563 polymorphism results across various populations may be mainly attributed to differences in sample size, as well as other genetic and environmental factors.

To expand our investigation, we assessed for the first time the association between seven ASD clinical variables and the selected SNP. Previously, variations in NRP2 have been reported to be associated with Attention-Deficit/Hyperactivity Disorder (*14*). Our results indicated that the NRP2 rs849563 polymorphism was significantly associated with rocking and swaying, with a p-value of 0.029 (Table 2). These behaviors are commonly observed in individuals with ASD and are associated with sensory processing issues and self-regulation difficulties, particularly in response to environmental overstimulation or emotional distress. Although these traits are not exclusive diagnostic criteria for ASD, they can serve as behavioral indicators, particularly when accompanied by other core symptoms such as repetitive behaviors and social communication difficulties. This significant association helps clarify the specific neural mechanisms contributing to these behaviors in individuals with ASD, underscoring the potential role of the NRP2 gene in modulating sen-

sory processing pathways implicated in autism symptomatology. Further research should also examine whether this SNP interacts with other genetic or environmental factors influencing sensory behaviors in ASD.

Our study did not find a significant association between the rs849563 polymorphism and other autism-related traits, including eye contact, repetitive behaviors, speech, cognitive understanding, echolalia, and response to name (Table 2). These findings suggest that while the NRP2 gene may contribute to certain aspects of ASD symptomatology, its influence on other core features of the disorder remains unclear. This could be attributed to the complex nature of the neural circuits involved in different behaviors. For instance, the amygdala and prefrontal cortex play crucial roles in social behavior and emotional regulation (*15*), while the striatum is linked to repetitive behaviors (*16*). It is possible that variations in other genes or environmental factors may interact with these CNS components to influence these behaviors (*17*). Understanding how NRP2 fits into this broader network of genetic and neural influences could provide further insights into the diverse manifestations of ASD.

One of the limitations of this study is the relatively small sample size, which may reduce the statistical power and limit the generalizability of our findings. A larger sample size would provide more robust results and improve the ability to detect smaller effect sizes. Another limitation is the statistically significant differences in age and sex between the patient and control groups. Future research with larger cohorts is needed to confirm the role of the NRP2 gene in ASD susceptibility and strengthen the evidence for the association between NRP2 rs849563 polymorphism and specific behaviors such as rocking and swaying. Nevertheless, this study makes an important contribution to the currently unexplored area of ASD genetics in the Azerbaijani population and lays the groundwork for future investigations involving larger cohorts and more comprehensive phenotypic assessments.

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