ORIGINAL ARTICLE

Psychiatry and Clinical Psychopharmacology 2023;33(1):14-19
DOI:10.5152/pcp.2023.22509

Peripheral Expression of *ADORA2A* Is Increased and Is Correlated with Autism Spectrum Disorder Severity in a Sample of Turkish Children

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ABSTRACT

Background: The aim of this study was to evaluate the peripheral expression of *ADORA2A* (Adenosine A2A receptor gene) in young subjects with autism spectrum disorder compared with healthy controls and its relationship with clinical characteristics.

Method: This study included 93 children and adolescents with a diagnosis of autism spectrum disorder as the study group and 105 healthy age- and gender-matched controls. Blood samples were obtained from all participants, and a real-time quantitative polymerase chain reaction was performed. Parent- and clinician-rated assessment instruments were used to assess and rate the severity of autism spectrum disorder and other emotional/behavioral problems.

Results: The mean age of the study group was 9.06 ± 3.57 and 86% were male (n = 83), whereas the mean age of the control group was 9.22 ± 3.86 and 86.7% were male (n = 91). We have found a higher level of peripheral expression of *ADORA2A* in children and adolescents with autism spectrum disorder compared with healthy controls (fold change = 1.33, $P = .001$). We also found a weak negative correlation with autism spectrum disorder severity ($r = -0.216$; $P = .038$) and stereotyped behaviors ($r = -0.207$, $P = .046$).

Conclusion: *ADORA2A* genes may have a role in the pathophysiology of autism spectrum disorder. Further studies are needed to evaluate whether peripheral expression of *ADORA2A* genes may be among the biomarkers for diagnosing or measuring the severity of autism spectrum disorder.

ARTICLE HISTORY

Received: August 31, 2022
Accepted: February 6, 2023
Publication Date: March 31, 2023

INTRODUCTION

Genetic factors play an important role in autism spectrum disorder (ASD) etiology,[1,2] tend to be diverse and heterogeneous (including chromosomal alterations, copy number alterations, single-gene disorders, polygenic mechanisms, and epigenetic alterations),[3] and show great variation among individuals.[4-6] For example, as of March 2020, 863 human genes have been linked with ASD in the Simons Foundation in Autism Research Initiative.[7] Among many other chromosomal regions, a region near the middle of chromosome 22, 22q11.2, deserves a particular interest in terms of ASD genetics. Various genetic alterations in this region, including deletions, duplications, and copy number variations, have been associated with ASD previously,[8,9] and genetic variants in this region are observed in approximately 1% of individuals with ASD.[10] Autism spectrum disorder accompanies 15%-50% individuals with 22q11.2 microdeletion syndrome.[10] One of the candidate genes within these loci, adenosine A_{2A} receptor gene (*ADORA2A*), is located on the 22q11.23 region and has been associated with various neuropsychiatric disorders, including ASD, attention-deficit/hyperactivity disorder (ADHD), depression, Tourette's disorder, panic disorder, schizophrenia, and Parkinson's disease.[11-16] *ADORA2A* encodes 1 of the 4 G-protein coupled adenosine receptors, adenosine A_{2A} receptor, and is expressed in various tissues, including the brain, liver, blood, and immune system.[17] In the central nervous system, *ADORA2A* has its highest expression in the striatum and modulates chemical neurotransmission by mediating the release of numerous neurotransmitters, including glutamate, dopamine, and

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Cite this article as: Akköprü H, Alnak A, Karadoğan ZN, Çağlayan AO, Özçetin M, Coşkun M. Peripheral expression of *ADORA2A* is increased and is correlated with autism spectrum disorder severity in a sample of Turkish children. *Psychiatry Clin Psychopharmacol.* 2023;33(1):14-19.

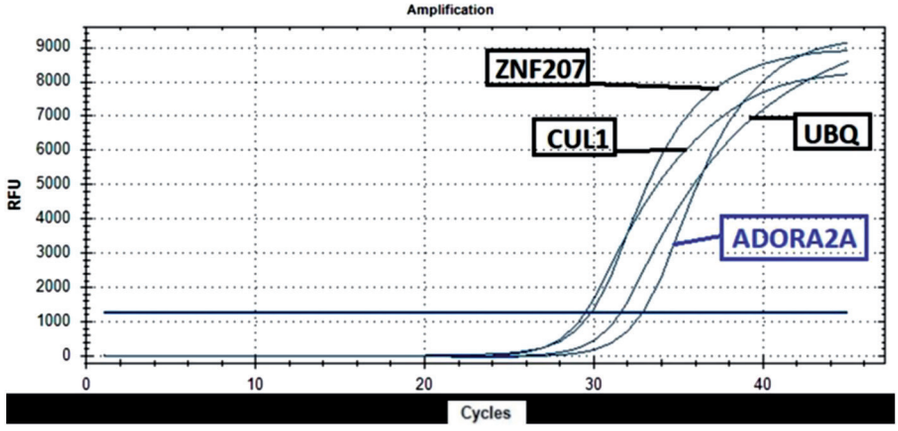

Figure 1. Representative image showing the peripheral gene expression profile of the housekeeping genes and *ADORA2A*.

γ-aminobutyric acid[18] (Figure 1). It is thought to have roles in mediating locomotion, sleep–wake cycle, fine-tuning of synaptic plasticity, glial activity, and neuroinflammation.[19,20] Epigenetic factors influence the expression of *ADORA2A* expression by controlling the promoter regions of the gene.[21] With respect to the psychiatric disorders, several *ADORA2A* single-nucleotide polymorphisms (SNPs) have been associated with depression,[16] anxiety,[12] ADHD,[15] Tourette's disorder,[14] and ASD.[11] In a case–control study by Freitag and colleagues,[11] 98 subjects with ASD and 234 controls were genotyped for 8 SNPs in *ADORA2A* and found a nominal association with ASD for the CC genotype of rs2236624. The authors also reported other SNPs to be associated with increased social interaction, nonverbal communication, and repetitive behavior.[11] Additionally, it has been shown that treatment with adenosine A_{2A} receptor agonists results in decreased stereotypic behavior in animal models of autism.[22]

However, despite the presence of several studies regarding *ADORA2A* SNPs or receptor genes in different psychiatric or neurodevelopmental disorders, there has been no study, to the best of authors' knowledge, investigating the peripheral expression profile of *ADORA2A* gene in subjects with ASD. Indeed, gene expression studies conducted in brain and/or peripheral tissues may provide further insight to understand the functional consequences of genetic variants which were previously identified as associated with ASD.[5,23] In the current study, we aimed to investigate the gene expression profile of the *ADORA2A* gene in a group of children and adolescents with ASD compared with healthy controls. We hypothesize that the *ADORA2A* expression profile would be different in children with ASD and would be correlated with the severity of ASD.

MAIN POINTS

- Level of peripheral expression of *ADORA2A* in children and adolescents with ASD was found to be higher when compared with healthy controls.
- There was a weak positive correlation with ASD severity and stereotyped behaviors.
- *ADORA2A* genes may have a role in the pathophysiology of ASD.

MATERIAL AND METHODS

The study group of this study was drawn from children and adolescents who have been followed up with the diagnosis of ASD at the Child and Adolescent Psychiatry Department of Istanbul University. A total of 93 children and adolescents aged between 2 and 18 years were included in the study. The diagnosis of ASD of the subjects was confirmed by the authors through detailed clinical examination based on diagnostic and statistical manual (DSM)-5 criteria. Exclusion criteria for the study were as follows: (i) patients having evidence of severe/profound intellectual disability, (ii) patients having a diagnosis of genetic, metabolic, or progressive neurological disease; and (iii) patients whose parents disagreed to participate in the study and rejected signing informed consent. The Childhood Autism Rating Scale (CARS) and Aberrant Behavior Checklist (ABC) were applied to evaluate symptom severity and/or accompanying behavioral and emotional difficulties. As the control group, age- and gender-matched children and adolescents without a history of any neuro developmental/psychiatric disorder (n=105) were recruited from the General Pediatric Outpatient Clinic of Istanbul University. They also underwent a detailed clinical examination by the authors to ensure they met the inclusion criteria. This study was approved by the Istanbul Medical Faculty Ethical Committee and supported by a grant from the Scientific Research Project Coordination Unit of Istanbul University (project ID no: TTU-2018-30570).

INSTRUMENTS

Interview Form

The interview form was developed by the authors and included questions on the patient's date of birth, gender,

contact information, and other basic sociodemographic information.

Childhood Autism Rating Scale

The CARS is a frequently used, valid, and reliable scale developed by Schopler et al[24] (1980) to assess disease severity and differentiate individuals with ASD and those with other developmental delays. It consists of 15 items, each assessing different aspects of ASD symptoms and development. The scale form adapted into Turkish was shown to be valid and reliable.[25]

Aberrant Behavior Checklist

Aberrant Behavior Checklist is a useful tool for evaluating inappropriate, problematic, and maladaptive behaviors associated with ASD or other developmental disorders and translated into more than 25 languages and is used commonly worldwide in subjects with ASD and developmental delay.[26] The Turkish form of ABC consists of 46 questions assessing 5 domains of behavior: hyperactivity/noncompliance, lethargy/social withdrawal, stereotypic behavior, self-injurious behavior, and other behaviors.[27]

Gene Expression Analysis

Total RNA was extracted from whole blood using Hybrid-R™ (GeneAll, Seoul, South Korea, catalog no: 315-150) and transcribed into complementary DNA with Ipsogen® cDNA Synthesis Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany, catalog no: 679923). The quantity, quality, and integrity of RNA were assessed on a Qubit 4 fluorometer (Thermo Fisher Scientific Inc., Wilmington, Del, USA). Real-time quantitative polymerase chain reaction was performed using 3 reference genes (*ABL-1*, *CUL1*, and *ZNF207)*. The primer sequences of the *ADORA2A* gene used for the amplification were (forward) 5′-CAT CTT CAG TCT CCT GGC CA-3′ and (reverse) 5′-ACC CAG CAG ATG GCA ATG ATG-3′. Relative changes in gene expression were analyzed with the ΔCT method.[28]

Statistical Analysis

R v3.4.0 and the Statistical Package for Social Sciences (SPSS), Version 21.0 (IBM SPSS Corp.; Armonk, NY, USA) were used for statistical analyses. Descriptive data were presented as mean and SD. Distribution of data was assessed using the Shapiro-Wilk test. Mann-Whitney *U*-test or independent samples *t*-test were used for comparison of continuous data according to the distribution of the data. Spearman's correlation coefficient was used for the comparison of non-normally distributed data. The chi-square test was used for the comparison of categorical data. The significance level was established as $\alpha = 0.05$.

RESULTS

The mean age of the study group was 9.06 ± 3.57 and 86% were male (n = 83), whereas the mean age of the control group was 9.22 ± 3.86 and 86.7% were male (n = 91). There was no significant difference in terms of age ($P = .763$) and gender ($P = .895$) between the study and control groups. There was no statistically significant difference between the 2 groups in terms of mother's ($P = .133$) and father's age ($P = .412$) at birth.

The mean CARS score of the study group was 41.98 ± 4.37 (33-51.50) and the mean scores of ABC subscales were 20.39 ± 9.01 for hyperactivity/noncompliance, 20.97 ± 9.89 for lethargy/social withdrawal, 6.28 ± 4.87 for stereotypic behavior, 1.43 ± 2.13 for self-injurious behavior, and 6.11 ± 3.14 for other behaviors. The CARS scores of the subjects were correlated with the total ABC scores ($r = 0.497$, $P < .001$) as well as with the subscales of ABC ($P < .001$). Sociodemographic and clinical characteristics of the participants are presented in Table 1.

Gene Expression Analyses

A representative image showing the peripheral gene expression profile of the housekeeping genes and *ADORA2A* is shown in Figure 1. When the groups were analyzed separately, the *ADORA2A* expression level was not different in terms of gender ($P = .851$ for the study group; $P = .373$ for the control group), and it was not correlated with age ($r = -0.66$, $P = .532$ for the study group; $r = -0.178$, $P = .70$ for the control group). When 2 groups were compared, the *ADORA2A* expression level was found to be increased 1.33-fold in the study group compared to the control

Table 1. Sociodemographic and Clinical Characteristics of the Study and Control Groups

	Study Group (n = 93)	Control Group (n = 105)	*P*
Gender (male/female) (n)	80/13	91/14	.895*
Age (years)	9.06 ± 3.57	9.22 ± 3.86	.763**
Mother's age at birth (years)	28.49 ± 6.45	27.17 ± 5.68	.133**
Father's age at birth (years)	32.16 ± 6.49	31.35 ± 7.15	.412**
CARS score	41.98 ± 4.37		
ABC scores			
Hyperactivity	20.39 ± 9.01		
Lethargy	20.97 ± 9.89		
Stereotypic behavior	6.28 ± 4.87		
Injurious behavior	1.43 ± 2.13		
Other behaviors	6.11 ± 3.14		
Total	55.17 ± 23.52		
ADORA2A[a] median (minimum-maximum)	3.42 (0.18-7.72)	2.88 (0.01-5.40)	<.001***

[a]Fold change = 1.33.
*Chi-square test.
**Independent samples *t*-test.
***Mann-Whitney *U*-test.
ABC, Aberrant Behavior Checklist; CARS, Childhood Autism Rating Scale.

Table 2. Correlations Between *ADORA2A* Expression Level and Phenotypic Characteristics (n = 93)

		CARS	ABC—Hyperactivity /Noncompliance	ABC—Lethargy / Social Withdrawal	ABC—Stereotyped Behavior	ABC—Self-Injurious Behavior	Other Behavior
ADORA2A	*r*	-0.0216	-0.035	0.089	-0.207	-0.157	-0.011
	*P**	.038	.741	.395	.046	.133	.917

*Spearman's correlation coefficient.
ABC, Aberrant Behavior Checklist; CARS, Childhood Autism Rating Scale.

group ($P < .001$). Median (minimum–maximum) values for *ADORA2A* expression for both groups are shown in Table 1.

Regarding the *ADORA2A* gene expression level and its correlations with different phenotypic characteristics of subjects with ASD, there was a weak negative correlation between the *ADORA2A* expression level and CARS score ($r = -0.216$; $P = .038$). Among the subscales of ABC, *ADORA2A* expression level was in a negative correlation with scores of stereotyped behaviors ($r = -0.207$, $P = .046$). No such significant correlation was observed for the other subscales of ABC ($P > .05$). Correlations between *ADORA2A* expression levels and phenotypic characteristics are presented in Table 2.

DISCUSSION

Gene expression studies in ASD can be conducted in the brain or peripheral tissues due to ethical and practical difficulties in obtaining brain tissue samples.[5,23] While brain gene expression data can also be used to identify potential biomarkers for the disease in peripheral tissues, generalization of findings from peripheral tissues could be more difficult.[23] Genes that show expression changes in the brain of subjects with ASD and are also dysregulated in peripheral tissue from ASD subjects are the most promising in this regard.[23] However, several independent studies have also shown consistency between peripheral and brain expression findings.[23] For example, *ITGB2* (integrin, beta 2) was found to be upregulated in ASD by 2 independent gene expression studies in peripheral tissues and was also found to be upregulated in the ASD brain.[23] Therefore, peripheral expression studies on candidate genes may expand our understanding of ASD genetics and provide a basis for future studies.

ADORA2A is located on the proximal region of the long arm of the chromosome 22, a locus that has attracted attention for decades in terms of ASD genetics.[10] This is the first study to evaluate the peripheral expression of *ADORA2A* in individuals with ASD compared with healthy controls. In line with our hypotheses, we have found a higher level of peripheral expression of *ADORA2A* in children and adolescents with ASD compared with healthy controls. We also found a negative correlation with ASD severity (as evaluated on CARS) and stereotyped behaviors (as evaluated on ABC).

Similar to our expressional findings, genetic variants in *ADORA2A* were found to be categorically associated with either ASD or its clinical phenotype.[11] In the study by Freitag et al.[11] 1 SNP in the *ADORA2A* gene (rs2236624) was represented in individuals with ASD and 3 others (rs3761422, rs5751876, and rs35320474) were found to influence the clinical phenotypes related to ASD.

ADORA2A is expressed in numerous quantities in both ventral and striatal structures.[17] The striatum is associated with restricted and repetitive behavior in ASD as well as social reward systems.[29] Striatal neurons expressing $A_{2A}R$ play mediating roles in dopaminergic and glutamatergic neurotransmission.[18] It is hypothesized that the increased expression of *ADORA2A* may facilitate the release of excitatory neurotransmitters, resulting in excitotoxicity.[21] Additionally, *ADORA2A* plays a regulatory role in neuroimmune development and blood-brain barrier permeability.[32] In human studies, abnormalities of *ADORA2A* expression have been found in several demyelinating diseases and spinal cord injuries.[32] Meanwhile, latest evidence indicated the role of neuroinflammation and immune system dysfunction in ASD pathophysiology.[33,34] In animal models of ASD, inactivation of the adenosine A_{2A} receptor is associated with a higher level of sociability in mice,[30] and the central expression of *ADORA2A* regulates the neuroimmune development and repetitive behavior of autistic mice.[22,31] In mouse models of 22q11.2 duplication syndrome, it has been shown that deletion of this chromosomal region (which includes *ADORA2A*) may interfere with the activity of the parvalbumin-producing interneurons. Given the role of neuroinflammation in ASD pathophysiology and the role of *ADORA2A* in neuroimmune development in human and animal models, one of the new findings in our study (which is negative correlation between *ADORA2A* expression and ASD severity) may be an interesting and important finding that may deserve further research.

Similarly, it is the first time in the literature that we reported a negative correlation between *ADORA2A* peripheral expression and stereotypic behaviors in human subjects with ASD. This finding may also be interesting, which is consistent with previous studies of animal models of ASD reporting that the central expression of *ADORA2A* regulates the neuroimmune development and repetitive behavior of autistic mice.[22,31] In our study, there was no significant difference in terms of age and gender between the study and control groups. Since the *ADORA2A* expression may be influenced by several sociodemographic variables,[35] it may be important to exclude gender and

age effects between the study and control groups. We also used more than 1 gene as normalizer (namely *UBQ*, *CUL1*, and *ZNF207*) in the procedure of gene expression quantification for the sake of increasing the accuracy of quantification.[36]

There may have been some limitations to our study. First, we used peripheral blood samples of the subjects as a source of biological material. Thus, we have evaluated the peripheral expression of the *ADORA2A*, which may not be predictive of genuine neural expression. Also, given that the changes in the brain tissue in ASD occur in the fetal period and in the early years of life, the gene expression levels in the age range of 2-18 years of our study may not reflect these periods.

In conclusion, a higher level of peripheral expression of *ADORA2A* was found in children and adolescents with ASD compared with their typically developing peers. We also found a negative correlation with ASD severity and stereotyped behaviors. As the first case–control study of *ADORA2A* gene expression in young subjects with ASD, our study may provide a basis for future studies in this area with the hope of searching for the biomarkers in the diagnosis and/or severity rating of ASD.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Istanbul University (Approval No: 05).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – H.A., M.C.; Design – H.A., A.A., M.C.; Supervision – M.C., A.O.Ç., M.Ö.; Funding – H.A.; Materials – H.A.; Data Collection and/or Processing – H.A.; Analysis and/or Interpretation – A.O.Ç., A.A.; Literature Review – H.A., A.A., Z.N.K.; Writing – H.A., A.A., Z.N.K.; Critical Review – M.Ö., M.C.

Declaration of Interests: The authors declare that they have no competing interest.

Funding: This study was supported by a grant from the Scientific Research Project Coordination Unit of Istanbul University (Project ID No: TTU-2018-30570).

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