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Peripheral Expression of *MACROD2* Gene Is Reduced Among a Sample of Turkish Children with Autism Spectrum Disorder

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ABSTRACT

Background: Genomic variations in mono-ADP ribosylhydrolase 2 (*MACROD2*) have been associated with autism spectrum disorder (ASD) in recent genome-wide studies and case reports. In this study, we aimed to evaluate the *MACROD2* expression profile in patients with ASD.

Methods: The study group included 100 children with a DSM-5 diagnosis of ASD, and the control group consisted of 105 healthy controls. Blood samples were obtained from all participants in this study, and the gene expression level was determined using quantitative reverse transcription PCR (RT-qPCR). Statistical analysis was performed with R 3.4.0 and Statistical Program for Social Sciences (SPSS for Windows, 21.0).

Results: The mean ages of the participants in the study and control groups were 9.22 ± 3.62 and 9.27 ± 3.86 years, respectively. There was no significant difference concerning gender (P = .944) and age (P = .914) between the 2 groups. *MACROD2* gene expression was found to be decreased in the study group compared to the control group (study group = 5.73, control group = 89.56; fold change =-3.967; P < .001). While the level of *MACROD2* expression was not correlated with the ASD severity, it was associated with the severity of the hyperactivity/impulsivity symptoms (P = .008).

Conclusions: This is the first study in the literature investigating the peripheral expression of the *MACROD2* gene. We showed that the expression level of *MACROD2* was decreased in patients with ASD when compared to the control group. As the relationship between the *MACROD2* gene expression profile and ASD remains to be further investigated, this study may provide an insight for further studies.

ARTICLE HISTORY

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in communicative/social skills and repetitive behaviors and/or restricted interests, with early onset. The prevalence of ASD has increased drastically over the recent decades and is currently estimated to be over 1%, and even higher in males.[1] Although the underlying pathological mechanisms and pathways involved in ASD have not been exactly clarified, there is compelling evidence in the literature suggesting that genetic factors constitute the most significant contributors. The genetic mechanisms underlying ASD are as complex and heterogenous as its clinical heterogeneity--from Mendelian inherited disorders to polygenic mechanisms and epigenetic alterations.[2] Although considerable progress has been achieved with cutting-edge sequencing technologies, the exact etiology of ASD remains unclear.

mono-ADP ribosylhydrolase 2 gene (*MACROD2*) contains 17 exons and encodes a 425-amino acid nuclear ADP-ribose glycohydrolase. The *MACROD2* protein deacetylates monoADP-ribose from target proteins.[3-5] Although its function is largely unknown, it is considered to have a significant role in the essential cellular processes including DNA repair, chromatin biology, regulation of gene expression, and long-term memory formation.[6]

Preliminary studies suggesting an association between the *MACROD2* gene and ASD were from a transgenerational genome-wide screening study conducted by Tsang and colleagues. The authors reported the region in which *MACROD2* is encoded as a candidate region for ASD.[7] In the following studies, the genetic alterations on this gene and their associations with ASD were studied in more depth.[8-15] One of the most consistently shown genetic variations, rs4141463, is located on the fifth intron of the

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gene, which is a hypersensitive area, and it is suggested that this single nucleotide polymorphism and others within its neighborhood could alter the expression of this gene.[8] Several studies have stated that the significance of genomic variations of *MACROD2* in ASD may show ethnic differences.[8-10] For example, in the study conducted by Anney and colleagues (2010),[10] the authors stated that association with rs4141463 was more significant in subjects with European ancestry. In a case-control study which included 1170 subjects with ASD and 35 307 controls from 5 different centers in Europe, researchers found supportive findings for rs4141463 in a German sample but not in English, Irish, Dutch, and Italian samples. In a study from Italy, researchers genotyped 233 probands, 423 parents, and 90 siblings from the Italian Autism Network and reported that they found no significant association between rs4141463 and ASD.[13] In a genome-wide study from Taiwan, researchers stated that they also observed an association with rs4141463 in the Taiwanese Han population.[12] In addition to genome-wide association studies, there is a case report of an autistic boy with a 388 kb deletion of a region located in the *MACROD2* gene.[16] There is also an identified deletion in the locus of *MACROD2* in Kabuki Syndrome, which is known to be accompanied by autistic traits.[3]

On the other hand, most of the studies mentioned above were mainly focused on investigating genetic variations at the DNA level, such as single-nucleotide polymorphisms and copy number variations (CNV). Nevertheless, the potential influences of these genetic variations at the level of trancription have not been investigated. In general, many of the genetic alterations reported to be associated with ASD only possess a small risk for the disease and tend to show great variations between individuals.[17] Moreover, a number of these variants show incomplete penetrance and may be localized in non-coding regions.[17,18] Thus, it may be useful to evaluate the transcriptional activity of a gene in which genetic variations have been previously identified. In a meta-analysis of gene expression studies in ASD, Voineagu and colleagues (2012)[19] concluded that the transcriptome analyses were more efficient than the DNA studies to identify differences between ASD and controls.

In this cross-sectional study, we aimed to investigate the expression profile of the *MACROD2* gene which has been linked with ASD by both GWAS and CNVs studies, in a group of young subjects diagnosed with ASD, compared to the typically developing controls, in Turkey.

METHODS

Participants

This study was conducted in Istanbul University's Istanbul Faculty of Medicine, Child and Adolescent Psychiatry Department, during the period between June and December 2017. Subjects for the study group were recruited from among the patients who were followed up with the diagnosis of ASD based on the DSM-5 criterion. One hundred young subjects aged 2 to 18 years old and who had a DSM-5 diagnosis of ASD were involved, upon obtaining parental written informed consent. Subjects were re-evaluated through comprehensive clinical examinations prior to the study and their diagnoses of ASD were confirmed by an experienced child and adolescent specialist. The Childhood Autism Rating Scale (CARS) and the Aberrant Behavior Checklist (ABC) were applied to assess symptom severity and/or other accompanying emotional and behavioral problems. While all CARS evaluations were performed by an experienced child and adolescent psychiatrist, parents were asked to complete the ABC questionnaire. We excluded the cases with severe or profound intellectual disability since the subjects with severe/profound intellectual disability have a higher possibility of being syndromic ASD (monogenic causative mutations, accompanying any metabolic syndrome, etc.) or of having accompanying structural brain pathologies. A diagnosis of severe/profound intellectual disability was given by the psychometric assessments of subjects, as well as by clinical examination. Thus, as the exclusion criteria, participants were required to be free of any severe/profound intellectual disability and previously known genetic, metabolic, or progressive neurologic diseases. The control group of the study was drawn from a population referred to a general pediatric outpatient clinic with the complaints of non-psychiatric symptoms. Age and gender-matched subjects were invited to participate this study. They had to be free of a diagnosis of ASD, intellectual disability, and previously known genetic, metabolic, or progressive neurologic diseases. The candidate subjects were also asked if they had ever been to a psychiatric unit or had thought that they needed psychiatric help at anytime during their lives. Subjects with no history of any psychiatric diagnosis and no complaints of any psychiatric symptoms were enrolled in the study. A total of 105 subjects were included as the control group upon obtaining parental written informed consent. An approval from the Ethics Committee of Istanbul University School of Medicine was obtained prior to the study (2017/748; June 23, 2017). This study was supported by a grant from Scientific Research Project Coordination Unit of Istanbul University (Project ID no. TTU-2017-26608).

INSTRUMENTS

Interview Form

The authors developed the interview form that was used in this study. In the interview form, there were questions to obtain information about patients' sociodemographic information.

Childhood Autism Rating Scale (CARS)

The Childhood Autism Rating Scale (CARS) is a 15-item questionnaire developed by Schopler et al.[22] (1980). It is a

widely used instrument with sound psychometric properties and is often utilized by researchers to assess ASD severity and to differentiate patients with ASD from patients with other types of developmental delays.[20-22] The study of the scale's Turkish adaptation revealed that the Turkish form of the scale is also valid and reliable.[23]

Aberrant Behavior Checklist (ABC)

The Aberrant Behavior Checklist (ABC) was developed by Aman and colleagues to evaluate and grade the intensity of inappropriate and maladaptive behaviors.[24] The Turkish form of the ABC has 46 questions with 5 subscales: Hyperactivity/Noncompliance, Lethargy/Social Withdrawal, Stereotypic Behavior, Self-injurious Behavior, and Other Behaviors.[25] Each question is rated on a 4-point scale, ranging from 0 (no problem at all) to 3 (severe problem). The Turkish version of the scale was studied by Sucuoglu et al.[25] and Karabekiroglu et al.[26]

Quantitative Reverse Transcription PCR (RT-qPCR) Study

The procedure of total RNA extraction from whole blood samples was performed using Hybrid-R™ (GeneAll, Seoul, South Korea, catalog no: 315-150) and products were then transcribed into complementary DNA from 1 μg of RNA with Ipsogen® cDNA Synthesis Kit, according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany, catalog no: 679923). The quantity and quality of RNA were evaluated on the Qubit 2 fluorometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). The following primer sets specific to *MACROD2* were designed with primer blast software (5′ to 3′): 5'-GGCTGTGATACTGGACATGC-3' and 5'-ATATGGCCCCTGGCTATTGG-3'.[27] All PCR amplifications were performed in 20 μL reactions containing 10 μL Real-time PCR Master Mix with EVAGREEN (GenMark, Turkey), 1 μL primer mix (10 pmol), 3 μL double-distilled water, and 5 μL sample DNA. All reactions were run on the CFX96 Real-time PCR device (Bio-Rad). Fragments were amplified using the following parameters: initial denaturation at 95°C for 15 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute (Supplementary Figure 1). Three different reference genes, *ABL-1*, *CUL1*, and *ZNF207*, were used to normalize the data. The specificities of the PCR products were assessed by the melting curve analysis. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method (−ΔΔCT value was used as logarithmic gene expression at the base of 2).[28]

Statistical Analysis

The Statistical Program for Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM SPSS Corp.; Armonk, NY, USA) and R 3.4.0 (Vienna, Austria) software programs were used for statistical analyses. The Kolmogorov-Smirnov test was used for assessing normal distribution of data. Descriptive data were presented as mean and standard deviation. Two different statistical analyses (*t*-test and Wilcoxon rank-sum test) were performed, and a *P*-value of less than .05 was considered to be statistically significant.

RESULTS

A total of 205 children and adolescents were included in the current study. The mean ages of the participants in the study and control groups were 9.22 ± 3.62 (2-17 years) and 9.27 ± 3.86 (3-17 years), respectively. Eighty-seven percent of the study group and 86.7% of the control group were male. There was no significant difference concerning gender and age between the 2 groups. There was no statistically significant difference between the 2 groups concerning mother's (t = 0.918; P = .349) and father's age (t = 1.754, P = .081) at birth. Consanguineous marriage was more frequent in the study group (26%) than the control group (13%) (P = .02). The mean CARS score of the study group was 42.19 ± 4.51, and mean scores of ABC subscales were: 20.50 ± 9.05 for Hyperactivity/Noncompliance; 21.12 ± 9.89 for Lethargy/Social Withdrawal; 6.49 ± 4.82 for Stereotypic Behavior; 1.59 ± 2.30 for Self-Injurious Behavior, and 6.15 ± 3.05 for Other Behaviors. The CARS scores of the subjects were correlated with the total ABC scores, as well as the subscales. The sociodemographic and clinical characteristics of the participants are shown in Table 1.

Quantitative Reverse Transcription PCR (RT-qPCR)

Gene expression of *MACROD2* was observed at measurable levels in 27% of the study group and 71.9% of the control group. All the 3 reference genes (defined as housekeeping genes) (*ABL-1*, *CUL1*, and *ZNF207*) were expressed in all samples in the present study. *MACROD2* expression was significantly less frequent in the study group than in the control group (chi-square value = 30.831; $P < .001$).

The findings obtained in this study suggest that *MACROD2* gene expression decreased in the study group by up to 4 to 5 times when compared to the control group. The salient results are presented in Table 2and shown as a box plot graphic in Figures 1and 2.

Among all the participants in this study, the mean age of subjects with *MACROD2* expression was significantly higher those in whom *MACROD2* was not expressed (10.45 ± 4.10 and 8.89 ± 3.36 for the groups with and without expression, respectively, mean difference = 1.55; P = .023, independent samples *t*-test) (Figure 3).

While the mean age of subjects with *MACROD2* expression was significantly higher than those without *MACROD2* expression (10.75 ± 4.30 and 8.87 ± 3.32 for the groups with and without expression respectively, mean difference = 1.88; P = .042, independent samples *t*-test) in the control group, this difference was not significant regarding the study group (10.24 ± 4.03 and 8.91 ± 3.42 for the groups with and without expression respectively, mean difference = 1.32; P = .137, independent samples *t*-test)

Table 1. Sociodemographic and Clinical Characteristics of the Subjects

	Study Group (n = 100)	Control Group (n = 105)	χ^2/t	*P*
Gender (male/female) (n)	87/13	91/14	0,005	.944*
Age (years)	9.22 ± 3.62	9.27 ± 3.86	0.107	.914**
Mother's Age at Birth (years)	37.55 ± 6.62	27.57 ± 5.08	195.28	.349**
Father's Age at Birth (years)	32.06 ± 6.46	30.56 ± 5.51	192.98	.081**
CARS Score	42.19 ± 4.51			
ABC Scores				
Hyperactivity	20.50 ± 9.05			
Lethargy	21.12 ± 9.89			
Stereotypic Behavior	6.49 ± 4.82			
Injurious Behavior	1.59 ± 2.30			
Other Behaviors	6.15 ± 3.05			
Total	55.85 ± 23.41			

*Pearson chi-square test; **Independent samples *t*-test.

We then performed correlation analyses to investigate any correlation between the expression level of *MACROD2* and total CARS score and ABC sub-scores (within the study group). A significant correlation was revealed between expression level and ABC Hyperactivity/Impulsivity subscale scores (R = 0.499, *P* = .08) (Figure 4). CARS (R = .026, *P* = 0.898), ABC Lethargy (R = 0.269, *P* = .175), ABC Stereotypic behavior (R = 0.080, *P* = .690), ABC Self-injurious (R = 0.072, *P* = .720), ABC other behaviors (R = 0.07 *P* = .720), and ABC total (R=0.344, *P* = .78). There were no significant differences between subjects with and without expression with regard to gender (*P* = .177), consanguineous marriage (*P* = .615) and having a relative diagnosed with ASD, intellectual disability (ID), or developmental delay up to the third-degree (*P* = .692).

DISCUSSION

In this study, we evaluated *MACROD2* expression levels of subjects with ASD compared to their age and gender-matched healthy peers, and found a four-to-five-fold decrease in the study group when compared to the control group. To the authors' knowledge, no previous study has evaluated the expression level of *MACROD2* in peripheral blood of subjects with ASD, relative to either with or without genetic variants possibly affecting its expression.

Table 2. Comparison of MACROD2 Gene Expression Level Between the 2 Groups

	Study Group	Control Group	Fold Change (log2(S/C))	*P*
MACROD2	5.73	89.68	−3.967	<.001*
MACROD2	1.73	59.59	−5.104	<.001**

*Independent samples *t*-test (subjects without gene expression are not included); **Wilcoxon Rank-Sum test (The value of 0 was assigned for subjects without gene expression).

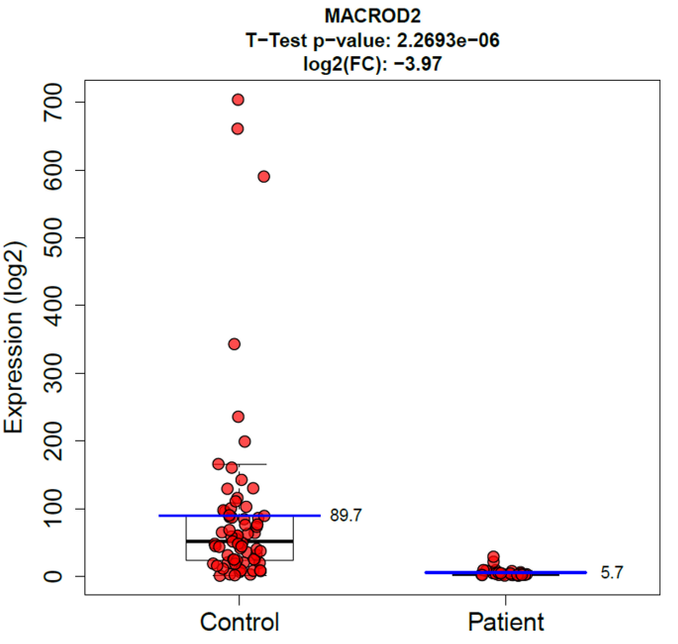

Figure 1. Boxplot graphic view of *MACROD2* expression distribution of patient and control groups when the independent samples *t*-test was performed. Subjects with no gene expression have not been included.

Regarding the genetic variants associated with ASD, Anney et al.[8], for the first time, linked the *MACROD2* gene with ASD by a genome-wide association study. After this

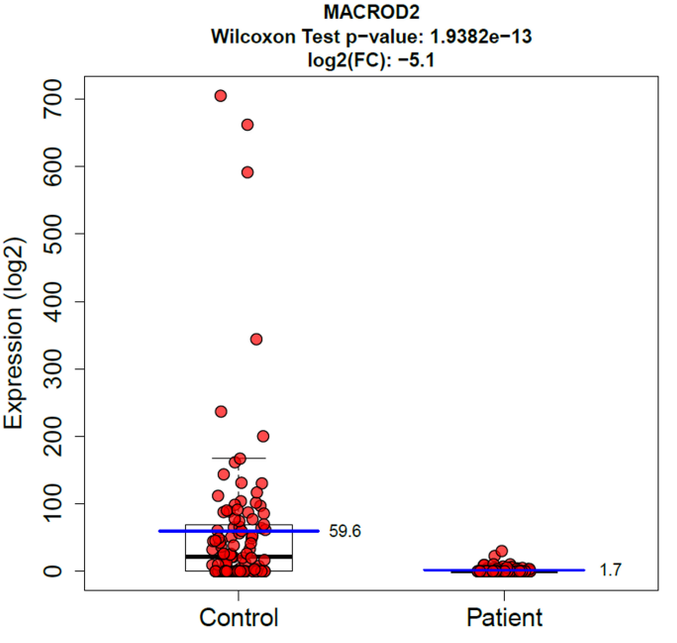

Figure 2. Boxplot graphic view of *MACROD2* expression distribution in patient and control groups when the Wilcoxon rank-sum test was performed. The value of 0 was assigned for subjects with no gene expression.

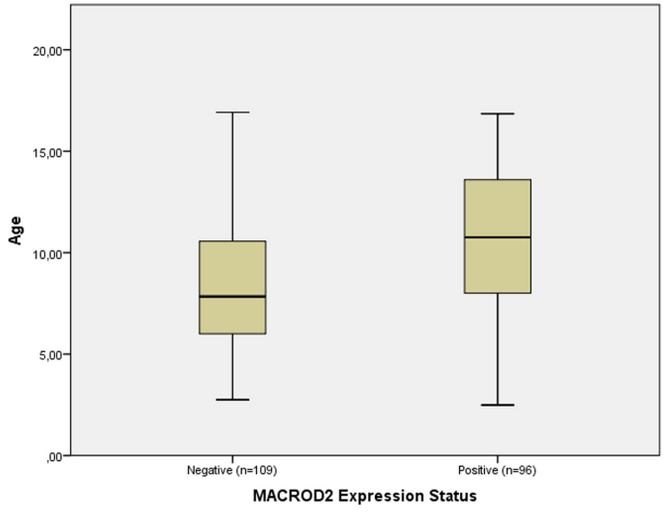

Figure 3. Comparison of participants according to their *MACROD2* expression status in terms of age.

initial report, replication studies were performed and positive[9,12,15,29,30] and negative associations[10,13,14] were found. Additional supportive findings[31,32] and case reports in patients with ASD and *MACROD2* mutations were reported.[3,16,33] After all, *MACROD2* is proposed to be a strong candidate gene for ASD by different large databases (SFARI)[34] or consortiums (Autism Genome Consortium).[29]

On the other hand, very little is known about functions of the *MACROD2* gene in cellular processes and the contributing mechanisms for various neuropsychiatric diseases during different developmental stages. While *MACROD2* is most commonly associated with ASD, it is also found to be associated with a few other neuropsychiatric diseases (attention deficit hyperactivity, schizophrenia, and depression),[35-38] brain infarct,[39] neurologic measures

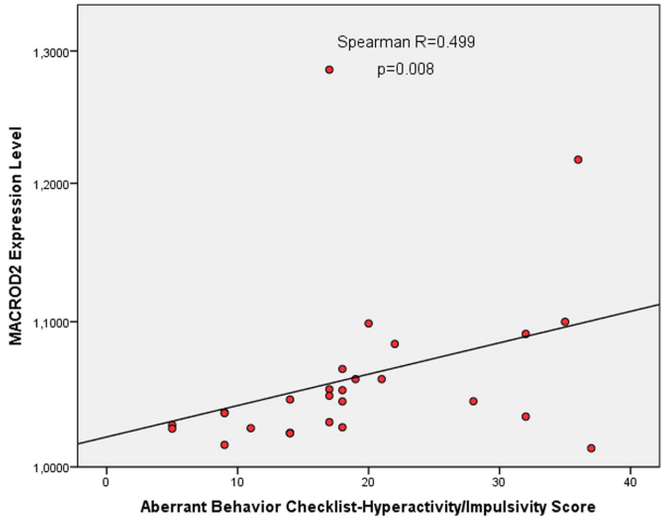

Figure 4. Correlation between *MACROD2* expression level and Aberrant Behavior Checklist-Hyperactivity/Impulsivity score.

(temporal lobe volume and brain connectivity),[40,41] and transient hydrops fetalis.[42] Regarding animal models, behavioral, morphological, and neurological phenotypes were shown in the *MACROD2* knock-out mice, which may also confirm a functional role of *MACROD2* in brain development.[6,43] In some studies, it is proposed that mutations on the *MACROD2* gene disrupt the organization of cytoskeletal structures and synaptic formations, which eventually lead to impairment of neural networks and brain connectivity.[40] Moreover, *MACROD2* plays an important role in chromatin formation and remodeling. By its metabolic sensing properties, it may change the chromatin dynamics and ameliorate the hazardous effects of cellular stress or inflammatory states.[44] While these explanations might be helpful to get an insight into the contributions of *MACROD2* to ASD etiology, potential pathogenetic mechanisms remain largely unknown.

There are no precise data on the levels of expression of *MACROD2* throughout tissues and life course. It is only known that the gene reaches its highest level of expression during the fetal period in the periventricular areas of brain.[10,11] To the best of the authors' knowledge, there are no certain data regarding the postnatal period. In our study, gene expression was found to be higher in the older children than in the youngers. While the implications of this difference remain unknown, our findings may provide a basis for further studies. On the other hand, the same age-dependent variation was not observed in subjects with ASD. This may further support our finding of altered gene expression in the subjects with ASD.

Before we further investigate the relationships of *MACROD2* expression and ASD severity or the accompanying emotional/behavioral problems, we wanted to optimize our clinical assessment data. While CARS is a commonly used instrument worldwide, it was developed more than 2 decades ago and it may not actually reflect the severity of ASD based on the DSM-5 criterion. On the other hand, it is reported that higher ABC scores are associated with more severe symptoms of ASD.[45] Consistent with these reports, there was a positive correlation between CARS and ABC scores in our study. This may suggest that our rating of symptom severity was accurate and provides reliable data to explore any association between symptom severity and gene expression level.

When the relationship between *MACROD2* expression and severity of ASD or accompanying behavioral or emotional problems was evaluated, *MACROD2* expression was not associated with ASD severity, as rated on CARS. This may vaguely suggest that *MACROD2* expression is not associated with overall ASD severity, but with some of the different domains of symptoms seen in ASD. Rather, among ASD subjects with detectable *MACROD2* expression, the severity of hyperactivity/impulsivity symptoms was moderately correlated with the gene's expression level. In a study, it has been shown that genetic alterations on

the *MACROD2* gene were linked with obesity associated with physical activity.[46] In another recent study conducted by Crawford and colleagues, *MACROD2* knock-out mice showed increased motor activity.[47] Moreover, diagnoses of attention-deficit hyperactivity disorder were reported in 2 males, both with exonal deletions in the *MACROD2* gene.[37] Along with our findings, it may be reasonable to consider a linkage between *MACROD2* gene function and activity level, either in the normative or pathological range of motor activity.

However, the significance of correlation in our study remains largely unknown since literature is scarce.

Our study has a few limitations. First, the sample size is small and replication studies are needed with larger cohorts. Second, results should be evaluated cautiously since the peripheral expression level of *MACROD2* is notably low. Third, gene expression is a tissue-specific phenomenon and peripheral expression of *MACROD2* may not reflect actual levels in neural cells. While evaluating the expression levels of a gene within the tissues assumed to be affected (ASD in this case) would definitely provide more certain data, sampling from the brain is almost always impractical in living individuals and sampling from post-mortem tissues also has its own disadvantages, such as a limited number of samples and poor RNA quality.[48] Moreover, it is also reported that gene expressions are correlated between peripheral blood cells and brain tissue at a moderate level.[49] Fourth, peripheral expression of *MACROD2* may not be predictive of genuinely neural expression.[48] We should note that because changes in brain tissue in ASD arise in the fetal period and 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. However, we should note that this is a limitation for expression studies in the relevant literature. Fifth, the genetic factors involved in the ASD etiology are complex and heterogeneous and vary considerably from one individual to another; the *MACROD2* gene and its functions may have contributed to the etiology of ASD in some of the patients included in our study. Because *MACROD2* expression was seen in only some of the subjects in the study group, we also compared subjects with *MACROD2* gene expression and those without expression concerning the CARS score, ABC total score, and parental age at birth. No significance for these variables was detected.

CONCLUSION

Genetic variations in *MACROD2* have been shown to be associated with ASD from several previous studies. As the first case-control study of *MACROD2* gene expression in ASD in the Turkish population, we found that the *MACROD2* expression is decreased in subjects with ASD compared to normally developing children. Given the lack of information on the expression profile of the *MACROD2* gene in different tissues through developmental periods, this case-control study may help in clarifying the current understanding of the potential consequences of mutations in the *MACROD2* gene for ASD.

Ethics Committee Approval: This study was approved by the Istanbul University Istanbul Medical Faculty Ethics Committee (2017/748; 23.06.2017).

Informed Consent: Detailed information about the study was given to the children and parents, and parental written informed consent was obtained from each participant and/or legal guardian.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept - A.O.Ç., M.C.; Design - A.O.Ç, A.A., M.C.; Supervision - A.O.Ç., M.C.; Resource - A.O.Ç., M.C., İ.K.O., A.A.; Materials - M.C., A.A.; Data Collection and/or Processing - İ.K.O., A.A.; Analysis and/or Interpretation - A.O.Ç., M.C., A.A.; Literature Search - A.O.Ç., A.A.; Writing - A.O.Ç., M.C., A.A.; Critical Reviews - A.O.Ç., M.C.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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Presented in: A part of the current research's results, with the same study population and different control samples, has been previously published as a part of conference proceedings (11th International Congress on Psychopharmacology & 7th International Symposium on Child and Adolescent Psychopharmacology held at Antalya/Turkey, 2019).

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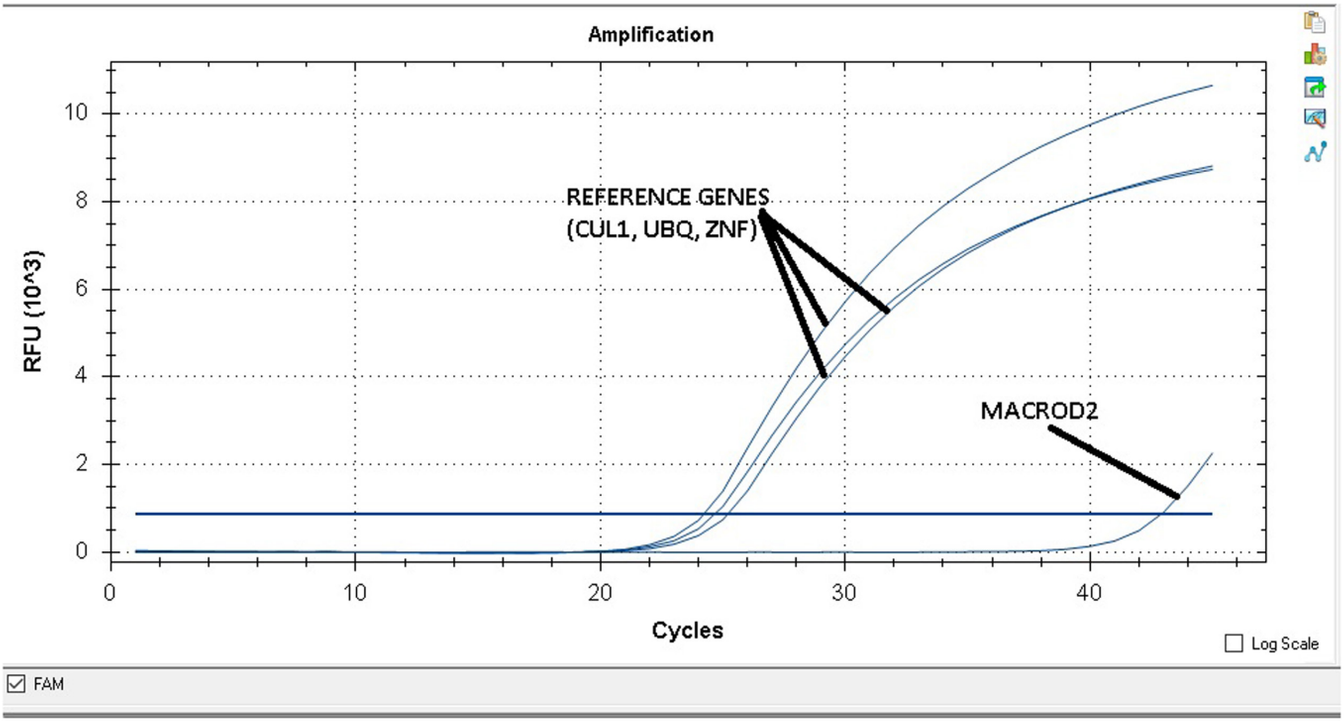

Supplementary Figure 1. Raw RT-qPCR image of the target and reference.