Candidate Gene Expression Investigation in Children With Attention Deficit Hyperactivity Disorder

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Research Article

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Abstract

Background: In this study, expression level analysis of genes associated with Attention Deficit Hyperactivity Disorder (ADHD) (*SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1,* and *ADRA2C*) by pre-treatment and post-treatment with Atomoxetine and Methylphenidate was investigated.

Methods: Forty-three ADHD diagnosed children and 38 healthy children were included to study. Forty-three patients with ADHD were divided into two groups, of which 35 patients used methylphenidate and 8 patients use atomoxetine. Five main study groups were generated: A control group, a group that includes methylphenidate pre-treatment samples, a group includes methylphenidate post-treatment samples, a group that includes atomoxetine pre-treatment samples and a group that includes atomoxetine post-treatment samples. Blood samples (10 ml each) were taken from everyone in study groups into EDTA tubes and RNA isolation was performed. mRNA expression levels of 11 determined candidate genes were showed via reverse transcription quantitative PCR method.

Results: The expression levels of *SLC6A3 (DAT)* of ADHD diagnosed children were significantly higher than the control group, while the mRNA expression levels of *SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT,* and *TPH1* genes were significantly lower (*t- test*, $p \leq 0.01$).

Conclusion: The expression level differences of these genes were determined to be useful as biomarkers in the diagnosis of ADHD. More patient numbers and studies with different groups are needed to fully reveal the relationship between these genes and the disease and its treatment.

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is an early onset, heterogeneous neuropsychiatric disorder that its etiology and pathogenesis are still largely unknown, incompatible with age and developmental level, manifests itself with symptoms of inattention, hyperactivity and impulsivity [1]. Studies conducted around the world have shown that the incidence of ADHD is between 1% and 20% and the prevalence is 5.29% [2]. ADHD prevalence was found to be 13.38% in a study conducted in Turkey [3]. It is observed with a higher frequency in boys. The male / female ratio in clinical and epidemiological samples was 9/1 and 4/1, respectively [4].

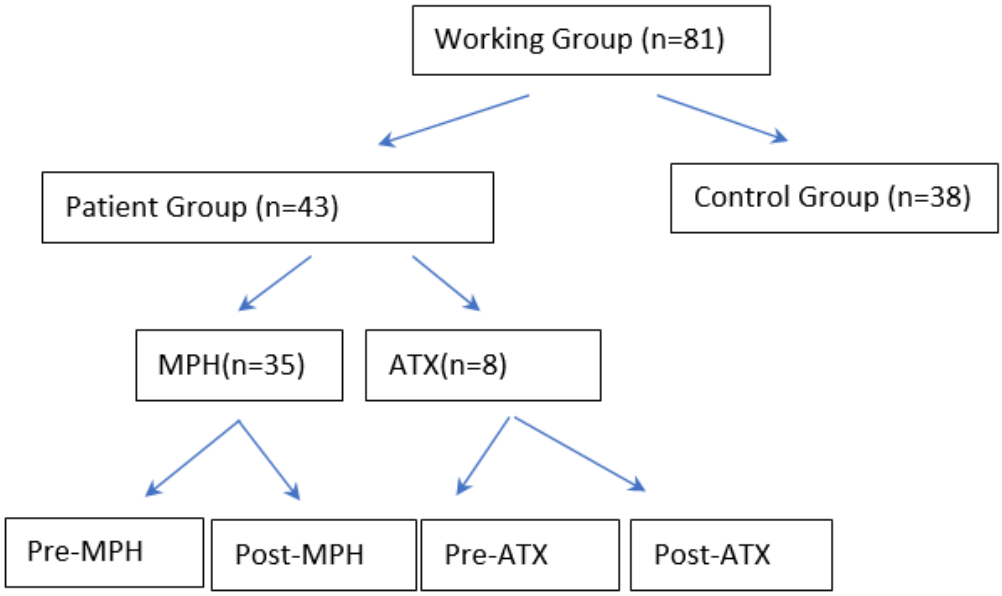
Many genetic, environmental, and biological factors play a role in the etiology of ADHD [5]. It was found that the prefrontal cortex, caudate nucleus, globus pallidus, corpus callosum, and cerebellum volume decreased in individuals with ADHD [6]. Genetic changes may affect cortical functions in the prefrontal area, dopamine level in the synaptic region, or dopaminergic receptor function [7]. Although the pathophysiology of ADHD states that the change in dopaminergic and noradrenergic pathways is not related to attention and impulse control, it is a heterogeneous disorder, but studies have focused on catecholaminergic norepinephrine (NE) and dopamine (DA) [7].

Drug treatments in ADHD are effective on NE and DA release in the prefrontal cortex. Stimulants such as methylphenidate (MPH) and non-stimulants such as Atomoxetine (ATX) are commonly used drugs in the treatment of ADHD. Psychostimulants act by preventing the reuptake of NE and DA from the synapse gap to the presynaptic neuronal space and increasing the release of monoamines into the extraneuronal space [8]. Most studies are based on related candidate genes, meta-analyzes, and genome-wide association studies (GWAS) show that dopaminergic, serotonergic and glutamatergic signaling, neuronal transmission, neuronal migration, and cell adhesion pathways play a role in the etiology of ADHD [9]. Therefore, the literature has been reviewed to identify potential candidate genes associated with neurotransmitters and *SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1* and *ADRA2C* genes in the neurotransmitter pathway were included in the study. The aim of our study is to investigate the role of these genes as potential disease markers by determining mRNA expression in whole blood and also to determine the possible effect of stimulant and non-stimulant drugs on the expression of these candidate genes.

Methods

Ethics committee approval was obtained from the ethics committee of Erciyes University Medical Faculty Hospital. Informed consents were obtained from all of the patients and their parents. The patients were selected among boys that are 6-12 years old who were diagnosed with Attention Deficit and Hyperactivity Disorder by an experienced psychiatrist according to DSM IV (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) diagnostic criteria and K-SADS-PL (Schedule for Affective Disorders and Schizophrenia for School-Age Children – Presentand Life timeVersion) criteria, who applied to the Pre-Interview Policlinic of Erciyes University Faculty of Medicine, Department of Child and Adolescent Mental Health and Diseases. Blood samples were taken from patients before 0.3-1 mg/kg of drug treatment was started. The patients were invited for a follow-up one month after the treatment had started, and blood samples were taken after the ADHD symptoms have been evaluated. Blood samples were taken for the control group from boys between the ages of 6-12, selected from the epidemiological area of the university hospital, who were examined by the same clinician and did not have any psychiatric or chronic medical diseases.

The patient group was divided into 2 groups as those using methylphenidate and atomoxetine. Blood samples were taken from these patients before and after the treatment to follow the expression differences of genes. The control group consisted of 38 volunteers.



RNA isolation and cDNA synthesis, leukocyte isolation was performed from samples taken from pediatric patients which are diagnosed with ADHD and control group cases (1xRBC LysisBuffer, Invitrogen). Leukocytes were taken into 1000µL TriPure reagent (Roche Applied Science, Basel, Switzerland) for RNA isolation and total RNA isolation was performed according to the protocol [10]. RNA concentrations and optical density measurements at 260 and 280 nm were determined by using the Nanodrop 1000 (ThermoFisherScientific, Germany) device for the quality and quantification of RNA samples. Purity was evaluated with a ratio of 260/280 nm. cDNA synthesis was performed by following the manufacturer's protocol with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) using random examer as primer, with 200ng total RNA in µl.

Quantitative Real-Time PCR analysis, from the obtained cDNA samples, the mRNA expression levels of *SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1,* and *ADRA2C* genes were investigated using the quantitative Real Light-Time PCR method and by the Cycler 480 II (RocheDiagnostic, Germany) device. Universal Probe Library (UPL) probe numbers that are specific to the cDNAs of the investigated genes are given in Table 1. Amplifications in 20µL total reaction volume; 10min 95°C and 45 cycles were performed in 10sec 95°C, 30sec 60°C, 10sec 72°C PCR program by using cDNA, mRNA-specific primers, UPL probe, LightCycler probes Master mix (Roche, Germany) and distilled water. The *Beta-actin* mRNA

expression level was taken as reference to normalize *SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH,1* and *ADRA2C* gene expression levels [11]. The experiment was repeated twice for each concentration. Expression levels of target genes were calculated according to the relative quantification method using the software program of the LightCycler 480 device.

Data analysis was performed with IBM SPSS Statistics 22 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Released 2013) software. In evaluating the conformity of the data to normal distribution; histogram, qq graphs, and Shapiro-Wilk test were used. The dependent sample *t-test* was used to compare data between two paired groups. Independent sample *t-test* was used to compare data between two independent groups. The relationship between variables was evaluated using Pearson's correlation analysis. The number of units (n) mean ± standard deviation values were given as summary statistics. $p < 0.05$ was accepted as the significance level**.**

Results

The mean age of the patients and control group were 8.88± 0.27, 9.11± 0.31, respectively. Among these patients, the mean age of those using MPH and ATX is 8.91 ± 0.31 and 9.54 ± 0.54, respectively. The average weight of the children in the patient and control groups was determined as 31.98 ± 1.58 and 35.18 ± 2.19, respectively. (Table 2)

The mRNA expression of *SLC6A3, SLC6A4, SLC1A2, VMAT (2), ADRA2C, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1* genes was determined by Real-time PCR. The *Beta-actin* gene was used as a housekeeping gene in the study and normalized values are given in Table 3.

When the pre-treatment patient group was compared to the control group, *SLC6A3* and DRD4 gene expression levels were found to be higher, but the increase in the *SLC6A3* gene mRNA was statistically significant. mRNA levels of *SLC6A4, SLC1A2, VMAT (2), MAOA, COMT, GLYAT, GRM5, ADRA2C, TPH1* genes were found to be decreased when compared to the control. This decrease in expression was statistically significant except for the *GRM5* and *ADRA2C* genes.

When the pre-MPH and post-MPH groups were compared, a decrease was observed in the *SLC6A3, COMT* and *DRD4* genes in the post-MPH group because of the treatment, and this decrease was significant only in the *SLC6A3* gene. A statistically significant increase was found in the expression of *SLC6A4, SLC1A2, VMAT (2), MAO, COMT, GLYAT, GRM5, ADRA2C, TPH1* genes in the post treatment group.

Pre- and post-ATX groups were compared, although there was a decrease in the expression of *SLC6A3, COMT* and *DRD4* genes in the post-ATX group because of the treatment, it was not found statistically significant.

An increase was observed in the expression of *SLC6A4, SLC1A2, VMAT (2), MAOA, GLYAT, GRM5, ADRA2C, TPH1* genes in the post-ATX group and this increase was found to be significant for *VMAT2, ADRA2C, MAOA, COMT, TPH1* genes ($p < 0.05$). If we assume the values in the control group to be normal, the expression levels of these genes in patients with treatment increased to normal values.When the patient group and the control group were compared after the treatment, no change was observed in the expression levels of all genes except *SLC6A3* and *COMT* genes. It was observed that the expression level of the *SLC6A3* gene in the patients decreased with treatment lower than that of the control group. Expression level of *COMT* was expected to approach the control group after treatment, but it decreased further with the treatment.

Correlation analysis were conducted within the control, pre- and post-treatment groups to determine how the relationship of genes expression is affected by ADHD. First, the correlation between the expression levels of genes in the control group was investigated. As a result of this research, it was determined that there is a statistically significant positive correlation between the expression levels of many genes. Correlation tests were conducted to determine whether the correlation between these gene expression levels was preserved and whether it was regenerated by the effect of treatment in the patient group after treatment.

A correlation was found between the expression of *SLC6A3* and *SLC6A4, MAOA, ADRA2C* genes in the control group (Table 4), and the correlation in these genes was not observed in the pre-treatment patient group (Table 5). There was a positive correlation as a result of treatment only in the *ADRA2C* gene (Table 6).

In the control group, a correlation was found between the mRNA levels of *SLC6A4* and *SLC6A3, SLC1A2, VMAT (2), GLYAT, GRM5, DRD4, TPH1* genes, and some of the correlation in these genes in the pre-treatment group (*SLC6A3, SLC1A2, GLYAT, DRD4*) was impaired in the post-treatment group (Table 5). It was observed that the correlation between the *SLC1A2, VMAT, GLYAT,* and *TPH1* genes was re-established because of the treatment (Table 6).

Discussion

Attention Deficit Hyperactivity Disorder is a childhood disease that affects all developmental stages of the individual, shows its effects throughout life, and shows significant functional impairment and high inheritance [12].

In this study, we aim to determine the role of candidate genes in the pathophysiology of ADHD by investigating the expressions of candidate genes that we think are involved in response to treatment. Most studies based on related candidate genes, meta-analyses, and genome-wide association studies show that dopaminergic, serotonergic and glutamatergic signaling, neuronal transmission, neuronal migration, and cell adhesion pathways play a role in the etiology of ADHD [13, 14]. Analyzes on ADHD sensitivity and MPH response in many meta-analyzes do not show consistent results [14]. Also, pharmacological studies on candidate genes could confirm *SLC6A3* as a key molecular target in drugs containing methylphenidate and atomoxetine in ADHD [15].

In our study, it was observed that the expression level of the *SLC6A3* (DAT) gene before treatment was doubled ($p \leq 0.001$) in patients compared to the control group. In addition, after methylphenidate and atomoxetine treatments, it was observed that the expression level of *SLC6A3* decreased compared to the expression level of the control group ($p \leq 0.05$). This shows that these drugs act by regulating the expression of this gene.

Grünblatt et al. studied the expression levels of the *SLC6A3* gene in 108 adult ADHD patients and 35 healthy controls. Accordingly, it has been observed that the expression level of the *SLC6A3* gene in patients is higher than in healthy individuals [16]. Our research on pediatric patients is consistent with the results of this study. With positron emission tomography, the amount of *DAT* in the internal globus pallidus (output nucleus of the basal ganglia) was high and this situation caused a decrease in dopamine levels; as a result, it was concluded that neuronal circuits that are effective in initiating behavior are affected and thus impulsive behaviors emerge [17]. Since DNA methylation silences gene expression by preventing transcription factors from binding to DNA, they hypothesized that the excess of *DAT* in the impulsive group is due to methylation in the binding site of a suppressor transcription factor (regulatory protein) that suppresses *DAT* expression (suppression of suppression) [18].

SLC6A4 gene reuptakes serotonin from the synaptic gap on the presynaptic membrane. Therefore, *SLC6A4* concentration in the membrane is one of the most important factors determining the amount of synaptic serotonin. Polymorphism in the promoter region of this gene affects the transcription rate of the transporter protein that performs the reuptake, hence the presynaptic *SLC6A4*, thus affecting the serotonergic system and mood [19].

While no difference was observed between the control and patient groups in the *SLC6A4* gene expression level study on adults [16], in our study on children, the expression level of the *SLC6A4* gene increased to the control group expression levels in patients. In addition, the expression level of the gene increased 3 times ($p \leq 0.001$) in patients after the use of methylphenidate, and the expression level doubled after the use of atomoxetine ($p \leq 0.05$).

In the expression study conducted by Sener et al. in children with autism spectrum, a significant difference was found in the patient group in terms of *SLC6A4* gene expression compared to the healthy control, parallel to our study [20]. Detection of lower expression in the patient group suggests a deficiency in serotonin reuptake. Allelic variants in the serotonin transporter

gene (*SLC6A4*), lower transcriptional efficiency, changes in serotonin concentration in various brain regions, and differences in *SLC6A4* mRNA expression have been associated with the development of ADHD [21].

SLC1A2 plays an important role in preventing extracellular glutamate concentrations from reaching neurotoxic levels and recycling glutamate at synapses by transporting glutamate to astrocytes to convert it to glutamine [22].

SLC1A2 (*EAAT2* or *GLT1*), which encodes glutamate transporter with high affinity especially in astroglial cells, is a brain-specific gene with a high degree of disorder. It is known that its expression changes in the glutamatergic system changes in the brain, especially in psychiatric disorders [23]. Decreases in the expression of this gene have been observed in many human and animal depression models [24]. The dysregulation of *SLC1A2* causes amyotrophic lateral sclerosis, Alzheimer disease, and epilepsy, as well as psychiatric disorders such as depression and autism [25]. In this study, the *SLC1A2* gene expression level was found to be significantly lower in patients than in the control group ($p \leq 0.001$). In addition, it was observed that the expression level of the *SLC1A2* gene significantly increased after methylphenidate and atomoxetine treatments ($p \leq 0.05$).

The vesicular monoamine transporter type 2 gene (*VMAT2*) has a very important role in the storage and synaptic release of all monoamines, including serotonin (5-HT). *VMAT2* level changes are associated with depression, bipolar disorder, and schizophrenia. In addition, studies show that changes in *VMAT2* levels cause Tourette syndrome, alcohol addiction, ADHD symptoms in children, and cognitive consequences after traumatic brain injuries in adults [26]. In studies conducted on mice models with *VMAT2* deficiency, dopamine intake and release into vesicles decreased more than 80%, pathophysiologically, dopaminergic adrenergic, cellular oxidative stress, alterations in alpha-synuclein accumulation and as behaviorally decreased in mobility, increased in depressive mood and sleep disturbances has been observed [26–28]. *VMAT2* over-expression resulted in an increase in uptake of dopamine into vesicles by 100% and its release into vesicles by about 80% and it resulted in increased mobility, anxiety and decreased depressive behaviors. High *DAT* and low *VMAT2* levels will theoretically result in cytosolic dopamine accumulation and minimal dopamine release. Low *DAT* and high *VMAT2* levels would theoretically result in low cytosolic dopamine content and high extracellular dopamine [29].

Our study showed that the *VMAT2* gene expression level of the control group consisting of healthy individuals was six times the expression levels of the patients ($p \leq 0.001$). In addition, it was determined that *VMAT2* expression in patients increased up to the expression levels of healthy individuals after methylphenidate and atomoxetine treatments ($p \leq 0.001$).

ADRA2C plays a role in the regulation of norepinephrine release from sympathetic nerves in the central nervous system in the adrenergic system. Noradrenergic neurons play a role in modulating wakefulness, regulation of visual attention, learning, and memory.[30].

According to the study conducted by Cho et al. on Korean patients with ADHD, a connection was established between *ADRA2C* (GT) repeat polymorphism and ADHD [31]. Barr et al. they worked on the same repeat polymorphism. Although Barr et al. could not establish a link between polymorphism and ADHD, they stated that other stronger SNPs in the *ADRA2C* gene are linked to ADHD and should be investigated [32].

In the expression study that we conducted on children, significant results were obtained between the expression levels of patients and healthy individuals. The *ADRA2C* expression level in healthy individuals was twice that of the patients ($p \leq 0.001$), and as a result of MPH ($p \leq 0.05$) and ATX treatment, *ADRA2C* expression in patients increased above the level of normal individuals.

Monoamine oxidase A (*MAOA*) is involved in breaking down monoamine neurotransmitters such as dopamine, 5-hydroxytryptamine (5-HT, serotonin), and norepinephrine. [33]

MAOA enzyme level is known to affect human behavior and characteristics. Some research has shown that a genetic polymorphism with low *MAOA* activity has an abnormal emotional response to environmental and social cues [34]. Additionally, a family study reported that *MAOA* enzyme activity is highly correlated with impulsivity. *MAOA* enzyme activity is known to be associated with the EcoRV polymorphism of the *MAOA* gene [35].

It has been reported that *MAOA* polymorphisms are associated with the hyperactive/impulsive ADHD type and the development of borderline personality disorder [36].

Weder et al. have found a correlation between exposure to moderate traumatic conditions during childhood with the low *MAOA* gene expression and risk of aggressive behavioral problems [37]. In this study, the expression level of the *MAOA* gene in healthy individuals was more than six times that of the patients ($p \leq 0.001$). *MAOA* expression levels of the patients increased significantly after MPH or ATX treatments ($p \leq 0.001$). Expression level in patients approached that of the control group.

Catechol O-methyl transferase (*COMT*) plays a role in the inactivation of catecholamines, including dopamine [38]. *COMT* gene has been seen as a focal point in studies on psychiatric disorders; SNP scans were performed, expression analyzes and protein studies were performed [39–42]. In the study conducted by Chen et al., all three parameters were evaluated. This study was conducted among healthy and schizophrenic patients, male and female individuals, and people of white and African descent, and provides a wide range of statistical results. No significant difference was found in *COMT* expression for age and disease parameters based on mRNA studies. Although the Val158Met SNP and the SNP in the 3 'end region are important risk factors for schizophrenia, the presence of these SNPs does not have a significant effect on mRNA expression. Researchers cannot explain the differences in protein studies and enzyme activities with mRNA expression and think that the functional effect of *COMT* has more complex genetic bases [42]. In another ADHD study, a general decrease in the surface area of the total premotor cortex was observed in males [43]. In our study, the *COMT* expression level was found to be lower in the patient group compared to the control ($p \leq 0.001$). In the patient group treated with methylphenidate, there was a decrease in the *COMT* gene expression level after the treatment, but it was not statistically significant ($p > 0.05$). A statistical decrease in the expression level was observed in the patient group after atomoxetine treatment ($p \leq 0.02$).

GLYAT encodes the enzyme Glycine-N-acyltransferase, which is responsible for metabolizing some metabolites in cells. Drugs are primarily metabolized to acyl-CoA intermediates. The glycine-N-acyltransferase enzyme catalyzes the combination of mitochondrial Acyl-CoAs with glycine [44]. Studies on the *GLYAT* gene on drug metabolism have been carried out, but they have not been focused on individuals with ADHD. In this respect, the significant results for *GLYAT* have great importance in this study. The *GLYAT* gene expression levels of the patients were lower than half the expression levels of normal individuals. ($P \leq 0.02$) In addition, the use of MPH brought the *GLYAT* mRNA level of the patients to the expression levels of normal individuals ($p \leq 0.01$).

Glutamate is the main stimulating neurotransmitter in the brain and plays a role in a number of ADHD-related processes such as brain development, modulation of neuronal activity, bidirectional regulation of dopamine signaling, synaptic flexibility, memory formation, and learning [45]. *GRM5* appears to be critical for inhibitory learning mechanisms because impaired receptor function causes inappropriate retention of deterrent memories that can lead to anxiety disorders [46]. Deletion in the CNV region of *GRM5*, one of the glutamate metabotropic receptor genes, has been associated with the presence of comorbid anxiety disorders [47].

In expression level studies conducted on patients with autism, it was observed that the *GRM5* expression level was low in these patients [48]. Our study was conducted on ADHD and it was observed that the *GRM5* expression level was lower in the patient group compared to the control group, as in children with autism, and also the use of MPH increased the *GRM5* expression level significantly ($p \leq 0.01$).

DRD4 is one of the dopaminergic system genes and one of the dopamine receptors that is most associated with ADHD. In the study conducted by Grünnblatt et al., *DRD4* and *DRD5* gene expression levels were found to be higher compared to patients [12]. The study we conducted supported this study and it was observed that the expression level of the *DRD4* gene was high in patients. But these results were statistically insignificant ($p > 0.05$). It was also observed that MPH and ATX treatments did not significantly alter *DRD4* gene expression levels.

Tryptophan hydroxylase 1 gene (*TPH1*) encodes a rate limiting enzyme in the biosynthesis of the monoamine neurotransmitter serotonin. Many studies have reported that *TPH1* and *TPH2* polymorphisms are associated with ADHD [49].

According to the study published by Taurines et al. (2011), no difference was found between *TPH1* gene expression levels, while according to a study published by Grünnblatt et al., (2012), expression level of the *TPH1* gene was higher in patient group than in control group [12, 16]. There are different results for many studies. As a result of our study, *TPH1* gene expression levels in patients were found to be statistically significantly lower than healthy individuals ($p \leq 0.01$). In addition, it was observed that MPH and ATX treatments increased *TPH1* expression levels ($p \leq 0.01$), closer to *TPH1* gene expression levels of healthy individuals.

There may be many reasons why the expression levels of the genes mentioned above differ between children with ADHD and the control group. These reasons may be variants in the regulatory regions of genes, as well as epigenetic regulatory mechanisms such as DNA methylation, histone modifications, and micro-RNAs seen in CpG islets in the promoter regions of these genes [16]. Therefore, post-transcriptional regulators activate and inactivate the translation of mRNA in some cases [49]. Although methylations generally create a silencing effect by suppressing the transcription of the gene, methylation of a regulatory region can sometimes lead to an increase in the gene product [18, 50].

In this study, the expression levels of genes (*SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1*), which are called candidate genes in the literature, differed between ADHD patients and the control group.

The *SLC6A3* gene expression level was found to be higher in children with ADHD compared to the control, and this elevation was reduced by medical treatments. ($p \leq 0.01$)

Expression levels of *SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, TPH1* genes were found to be less in children with ADHD compared to the control, and this decrease was increased with medical treatments. ($p \leq 0.01$)

As a result, with this study, we reveal that these genes are potential molecular biomarkers that can be used to diagnose ADHD. Improvement was observed in children whose ADHD status was evaluated according to the post-treatment scale. Both the differences in expression between patients and control groups and the correction of these differences with treatment show that the studied genes are biomarkers in the diagnosis of ADHD and the monitoring of treatment. Future studies aim to further investigate these genes in a larger independent population, including different ADHD subgroups (inattentive, hyperactive, and compound type) that are unresponsive to treatment. We aim to obtain more specific sets of biomarkers to compare these not only with healthy controls, but also with other psychiatric disorders, and to distinguish between childhood, adolescent and adult forms of each sub-disorder and ADHD. In addition, miRNAs known as post-transcriptional regulators that target these candidate genes can be investigated and can help find therapeutic molecular agents that target factors that cause the suppression or degradation of the mRNA of these genes.

Declarations

Acknowledgments

This study conducted as a part of project TSD-12-4112 of Erciyes University, thesis for phD.

Conflicts of interest

We hereby declare that we have no conflict of interest related to the manuscript. Ethics committee approval was obtained from the ethics committee of Erciyes University Medical Faculty Hospital with the number TSD-12-4112 dated 28.07.2017.

The study is supported by Erciyes University project TSD-12-4112, thesis phD.

We hereby declare that we have no conflict of interest related to the manuscript.

Ethics committee approval was obtained from the ethics committee of Erciyes University Medical Faculty Hospital. Informed consents were obtained from all the patients and their parents.

Corresponding author has availability of data and material.

Informed consent was obtained from all individual participants included in the study.

Patients signed informed consent regarding publishing their data and photographs.

Author Contrubition

HA and YE developed the theory and concept of the study. YO and MD designed the study. MD, NG, SO, and MED investigated and supervised the findings of this work. HA wrote the manuscript with support from NG, SO, MED, YO and MD. NG and HA performed the statistical analyzes. MD, HA and SO verified the analytical methods. HA, NG, and YE made literatüre search. MED, SO and HA performed clinical studies. SO analyzed and interpreted the patient data. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

Informed consent and consent of participants

This is a research study, and we aim to determine the genetic changes that are thought to occur with treatment in the candidate genes of ADHD patients. By identifying these changes, we aim to contribute to genetic screening programs and genetic counseling for your next generations.

First, we will determine the volunteers to be included in the project from among the patients who have been diagnosed with ADHD according to the diagnostic criteria of K-SADS (Schedule for Affective Disorders and Schizophrenia for School Aged Children - Present and Lifetime Version) in the Department of Child and Adolescent Mental Health and Diseases at Erciyes University. If you volunteer in this study, you will be examined by a detailed clinical evaluation at the Department of Medical Genetics at Erciyes University. If any other ADHD-related illness is detected, you will be removed from the study (such as Tourette's syndrome, Fragil X, Autism etc.). A detailed family tree of each volunteer participating in the study will be prepared and each person will be given a number. For this research, we need to draw about 10 ml of blood from each volunteer. RNA will be obtained from this blood as genetic material. After your blood is taken, the process of obtaining your RNA will be carried out in the laboratories of our department in accordance with international standards. Genetic screening will be performed in the center determined domestically from the obtained RNA samples, and genetic changes in target genes will be determined in these patients by analyzing the data. You will not be charged any fees at any stage of these transactions. During the blood collection, you may feel a little pain due to the needle stick, and in a very unlikely event, there may be a prolonged bleeding or infection risk after the needle stick.

Possible risks of genetic testing: Social, economic, and psychological problems may occur depending on the use of genetic information. We will do our best to ensure that your genetic information will remain confidential. However, because of the tests performed, it may be revealed that you or a member of your family may be affected by this disease in the future. The misuse of this information may affect you negatively in economic and social terms, and the illness may also affect you psychologically. It will only be with your permission to share the information of the research with someone other than you. Another important risk of genetic testing is the determination of the biological identity of the mother or father. In these cases, the principle of confidentiality will also be adhered to.

These are the potential risks that can be experienced in research. However, we will do our best to avoid any damage.

You may refuse to give biological material to obtain your RNA. This is entirely at your discretion. Let us emphasize that: You have the right to withdraw your consent by requesting the destruction of your RNA, WHEN YOU WANT WITHOUT ANY REASON. In such a case, your RNA will be destroyed as soon as possible, and all your records will be deleted.

The records that will reveal your identity in this research will be kept confidential, will not be disclosed to the public, and your identities will remain confidential even if the research results are published.

The following information must be provided in order for this form to be processed accurately. Patients have the right to refuse to sign this consent form; refusal to sign this form will not affect their care in any way. I hereby give my consent for images or other clinical information relating to my case to be reported in a medical publication. I understand that my name and initials will not be published and that efforts will be made to conceal my identity, but that anonymity cannot be guaranteed. I understand that the material may be published in a journal, Web site or other form of publication. As a result, I understand that the material may be seen by the general public. I understand that the material may be included in medical books.

Name of the patient

_____

Signature of patient (or signature of the Date Person giving consent on behalf of the patient) If you are not the patient, what is your relationship to him or her? (The person giving consent should be a substitute decision maker or legal guardian or should hold power of attorney for the patient _____

Why is the patient not able to give consent? (e.g. is the patient a minor, incapacitated or deceased?)

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Tables

Table1. Primer sequences used in Realtime PCR

Gene	Assay ID	Primers (5'-3')	UPL No
DAT1, SLC6A3	143278	F;CAGACACCGTGAGCTCTTCA R;CAGGAGCGTGAAGACGTAGAT	69
SERT, SLC6A4	112029	F;AAGTTCAACAACAACTGCTACCAA R;GAAGCTCGTCATGCAGTTCA	37
SLC1A2	111792	F;CCTGCCAACAGAGGACATC R;GACTGAAGTTCTCATCCTGTCCA	76
VMAT(2)	112188	F; CGAACTTTGGAGTTGGTTTTG R;CCCATGATAGGCATCATTGAC	138
MAOA	113315	F;GGAGGTGGCATTTCAGGAC R;AAAACCAAAACACTAACGCCATA	74
COMT	112923	F;GTCTTCCTCGACCACTGGAA R;TCACGTTGTCAGCCAGTAGC	40
GLYAT	118559	F;TGGCCTGATTTTAATACAGTGG R;TTGGTATAGTGATCAAGGTCATCTG	15
GRM5	100941	F;CCTGCCAACAGAGGACATC R;GACTGAAGTTCTCATCCTGTCCA	7
DRD4	112907	F;CAGACTCCACCGCAGACC R;GTGATGTGCACCACGAAGAA	4
TPH1	113010	F;GGACTTATAAAAGCCCTGAAAATCT R;TTCGGGACTCGATATGTAACAG	2
ADRA2C	145162	F;CAGGAGCTTGGCAGAGAGAT R;GAAGGCAAAGGGGTCTCC	1
B.ACT	101125	F; GGCCAGGTCATCACCATT R;GGATGCCACAGGACTCCAT	11

Table 2. Age and weight information of the patient and control groups

	Age (mean, SS)	Weight (mean, SS)
Patients	8.88 ± 0.27	31.98± 1.58
Patients treated with MPH	8.91 ± 0.31	31.80± 1.98
Patients treated with ATX	9.50± 0.54	32.75± 1.73
Control	9.11 ± 0.31	35.18± 2.19

Table 3. Normalized expression values of candidate genes

Genes	Control (n=38)	Pre- Treatment (MPH+ATX) (n=43)	Post Treatment (MPH+ATX) (n=43)	MPH		ATX	
				Pre-MPH (n=35)	Post-MPH (n=35)	Pre-ATX (n=8)	Post-ATX (n=8)
SLC6A3	9.89±1.55	20.34±2.29	4.41±0.43[a]	20.42±2.11	4.58±0.50[b]	20.00±8.67	3.68±0.73
SLC6A4	1.32±0.13	0.56±0.07[a]	1.42±0.15	0.55±0.07	1.53±0.18[b]	0.57±0,2	0.93±0.27
SLC1A2	2.47±0.49	1.04±0.12[a]	2.54±0.24	0.10±0.12	2.78±0.27[b]	1.22±0.35	1.51±0.30[c]
VMAT2	3.88±0.37	0.61±0.07[a]	4.25±0.45	0.62±0.08	4.53±0.54[b]	0.54±0.14	3.06±0.42[c]
MAOA	2.75±0.93	0.42±0.04[a]	1.89±0.28	0.41±0.03	1.93±0.35[b]	0.47±0.09	1.73±0.36[c]
COMT	1.18±0.07	0.80±0.07[a]	0.54±0.06[a]	0.74±0.07	0.55±0.08	1.03±0.18	0.51±0.10
GLYAT	1.76±0.22	0.78±0.21[a]	1.87±0.20	0.51±0.12	1.80±0.21[b]	1.94±0.94	2.23±0.53
GRM5	6.1±1.5	3.22±0.54	10.4±2.33	3.41±0.12	10.41±2.64	2.42±1.41	10.36±5.2
DRD4	1.8±0.13	3.06±0.97	1.83±0.42	3.04±1.13	1.76±0.51	3.14±1.66	2.11±0.48
TPH1	5.36±0.7	1.44±0.15[a]	4.31±0.59	1.47±0.19	3.72±0.54[b]	1.33±0.18	6.88±1.92[c]
ADRA2C	5.21±1.62	2.18±0.72	7.37±1.85	2.46±0.87	7.01±2.18[b]	0,93±0,47	8.93±3.11[c]

[a] The gene expression levels between the control and the patient groups were statistically significant (*p<0.05*)

[b] The gene expression levels between pre- and post-MPH treatment groups were statistically significant (*p<0.05*)

[c] The gene expression levels between pre- and post-ATX treatment groups were statistically significant (*p<0.05*)

Table 4. Correlation results of inter-gene expression values in the control group

	SLC6A3	*SLC6A4*	*SLC1A2*	*VMAT2*	*MAOA*	*COMT*	*GLYAT*	GRM5	DRD4	*TPH*1	ADRA2C
SLC6A3		b			a						a
SLC6A4	b		b	a			a	b	b	a	
SLC1A2		b									
VMAT2		a					a			a	
MAOA	a										
COMT											
GLYAT		a		a						a	
GRM5		b									
DRD4		b								b	
TPH1		a		a			a		b		
ADRA2C	a										

a *p<0.01*

b *p<0.05*

Table 5. Correlation results of pre-treatment gene expression values in the patient group

	SLC6A3	*SLC6A4*	*SLC1A2*	*VMAT2*	*MAOA*	*COMT*	*GLYAT*	*GRM5*	*DRD4*	*TPH1*	*ADRA2C*
SLC6A3									a		
SLC6A4				a				b		b	
SLC1A2				b	b	a			a	a	
VMAT2		a	b		b		a	a	a	a	
MAOA			b	b							
COMT			a				a		a	a	
GLYAT				a		a			a	a	
GRM5		b		a						a	
DRD4	a		a	a		a	a			a	
TPH1		b	a	a		a	a	a	a		
ADRA2C											

a *p<0.01*

b *p<0.05*

Table 6. Correlation results between post-treatment gene expression levels in the patient group

	SLC6A3	*SLC6A4*	*SLC1A2*	*VMAT2*	*MAOA*	*COMT*	*GLYAT*	*GRM5*	*DRD4*	*TPH1*	*ADRA2C*
SLC6A3						b					b
SLC6A4			a	a	b		a			a	
SLC1A2		a									
VMAT2		a			a					a	
MAOA		b		a						a	
COMT	b								b		a
GLYAT		a							a	a	
GRM5											
DRD4						b	a				a
TPH1		a		a	a		a				
ADRA2C	b					a			a		

a *p<0.01*

b *p<0.05*

Figures

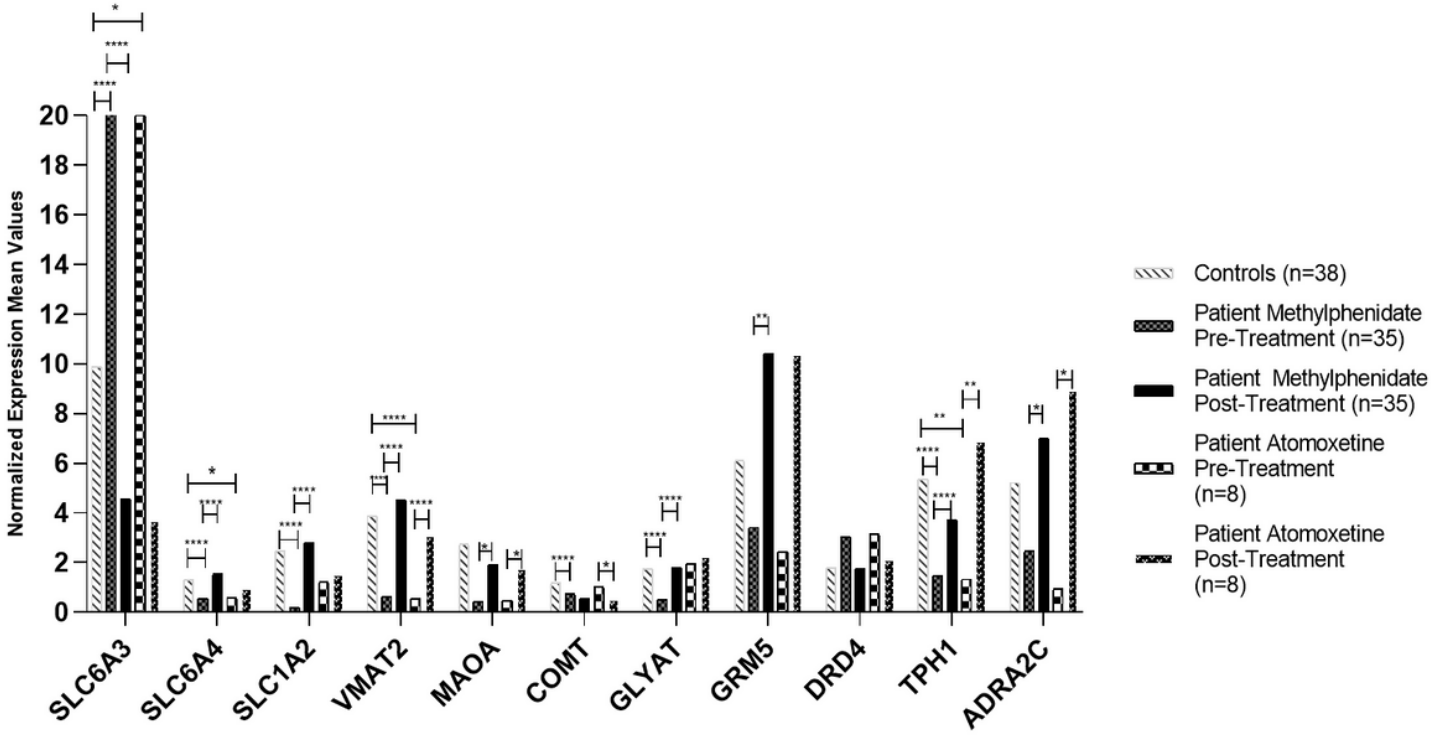

Figure 1

mRNA expression of intergroup genes in control, pre- and post-treatment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001