Molecular Analysis of STin2 (Intron 2) Variant of The *SLC6A4* Gene in Children and Adolescents With Attention Deficit Hyperactivity Disorder

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Abstract

Background

Attention deficit hyperactivity disorder (ADHD) is recognized as one of the most familiar childhood psychiatric disorders. Many molecular genetic reviews suggest that genes play a crucial role in susceptibility to ADHD. The serotonin transporter gene (*SLC6A4*) has polymorphisms that seem to correlate with ADHD development. The association between ADHD and the *SLC6A4* gene variants in the Iranian population has not been investigated yet. This study analyzes the STin2 (intron 2) variant of the *SLC6A4* gene in Iranian children and adolescents with ADHD .

Materials and Methods

In this retrospective case-control study, 86 ADHD patients and 99 healthy volunteers aged 5 to 14 years old were enrolled as the case group and the control group, respectively. The STin2 (intron2) fragment of the *SLC6A4* gene was amplified using specific primers by conventional PCR, and three STin2 alleles of the *SLC6A4* gene (STin2.9, STin2.10, and STin2.12) were examined using the acrylamide gel method.

Results

We found no significant difference between the ADHD and the control groups in STin2.9(34.9% vs 39.4%, p -value = 0.824), STin2.10(29.1% vs 23.2%, p -value = 1.354), and STin2.12(36% vs 36.4%, p -value = 0.986) variants.

Conclusion

It is concluded that there was no association between the frequency of STin2 variant alleles of the *SLC6A4* gene and ADHD, but in the study of risk estimation, it was found that allele 10 of this variant is a risk allele in ADHD patients.

1. Background

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders in children, with a worldwide prevalence of 4–8% in school-matured youngsters and may continue during adulthood in 50–80% of cases (1–3). ADHD is described by symptoms of inattention, hyperactivity, and impulsivity and has a wide range of possible clinical presentations(4&5). Considering the importance of ADHD in children and its impact on their health and the fact that its exact etiology is not yet completely understood, evaluating and researching the possible genetic factors involved in it may help us to comprehend, prevent, and possibly cure this disorder better.

To find better ways to treat and decrease the risks of ADHD, scientists are researching cause(s) and risk factors. Although the etiology and risk factors for ADHD remain unclear, recent evidence suggests that genetics play a key role (6–10). The ADHD patients' genes have been evaluated and statistically, among these genes, *DRD4*, *DRD5*, *DAT*, *DBH*, *SNAP-25*, *HTR1B*, and *5-HTT* seem to be involved in the etiology of ADHD (2 & 11–14).

The location of the serotonin transporter gene (*SLC6A4*; *5HTT*) is on chromosome 17q. This gene encodes a carrier protein responsible for retaking serotonin from the synapse and returning it to presynaptic neurons, which has a crucial role in serotonergic activity regulation within the brain. Attention, memory, and voluntary activity are connected to areas of the brain such as the amygdala, hippocampus, thalamus, putamen, and anterior cortex, which are the areas that *5HTT* is expressed (12).

Based on the available evidence, the *5HTT* gene could play a significant role in ADHD. Studies have observed this gene's role in impulsivity's etiology and stimulus responses in hyperactivity (14).

The serotonin transporter gene (*5-HTT/SLC6A4*) is amongst the most researched genes in psychiatry and has been linked with a wide variety of diseases (11).

A study reported a significant association of the polymorphism within the promoter region of *5-HTT* with scores on the Wender Utah Rating Scale, which is used to assess a history of ADHD-associated symptoms, indicating a higher frequency of the long variant allele in individuals with high scores (15).

One common polymorphism of the *SLC6A4* gene is STin2 VNTR (Serotonin Transporter Intronic VNTR Enhancer), a 17-bp variable number of tandem repeats. Two primary alleles (called STin2.10 and STin2.12) and additional low-frequency alleles (called STin2.7 and STin2.9) are involved in this polymorphism (16).

Although some studies have reported the STin2.12 allele (a major allele of STin2 polymorphism) as a transcriptional enhancer, one research showed that STin2.12/STin2.12 homozygotes appear to show fewer serotonin transporters available inside the brain (16–18). In 2002, Zoroğlu et al. noted that the STin2.12/12 variant of VNTR polymorphism appears to be associated with an increased risk of ADHD (19).

In a study that evaluated the association between ADHD and polymorphism of the two regions of the *5-HTT* gene [variable number of tandem repeats (VNTR) and *5-HTTLR*] *5-HTTLPR* S/S genotype was significantly lower in the ADHD group but Homozygous and heterozygous L variant predominated in it. The VNTR STin2.12/12 genotype was found significantly less in the ADHD group but there was no significant difference between the frequency of the short (S), long (L), 10, and 12 alleles in the two groups. They suggested the lack of an S/S variant of *5-HTTLPR* polymorphism or the STin2.12/12 variant of VNTR polymorphism as a risk factor for ADHD (19).

In a study evaluating the serotonin transporter gene in aggressive children with and without ADHD, the 10R allele of the *5HTT* VNTR polymorphism was significantly less frequent in the study group and there

was a significant link between *5HTTLPR* and ADHD. Aggressive children were statistically more likely to have at least one copy of the long allele than were those without ADHD (20).

In a study evaluating the possible role of the *5-HTTLPR* polymorphism in childhood disruptive behaviors using the haplotype relative risk design, a significant decrease in the short/short 5-HTTLPR genotype was observed in the ADHD type III combined group. Comparing the allele frequencies yielded similar results (21).

A review article reports that when the *5-HTTLPR* studies are combined, the pooled OR for the long allele is 1.31 (95% CI 1.09–1.59) (3).

Although there are many studies about ADHD, few have focused on the role of STin2 variants. Therefore, we aimed to analyze the molecular analysis of the STin2 variants of the *SLC6A4* in children and adolescent with ADHD.

2. Methods

2.1. Samples

The study group consisted of 86 children from Northwest area of Iran, who were diagnosed with ADHD by the Diagnostic and Statistical Manual (DSM- 5) criteri (22). The control group consisted of 99 non-psychiatric participants with similar demographic features such as mean age and gender and were referred to the children's hospital affiliated to Tabriz University of medical sciences for adenotonsillectomy and required routine lab tests. The sampling of target members was subject to the psychiatrist's convenience sampling technique based on inclusion and exclusion criteria. Additionally, participants' parents filled up informed consent.

This study's confirmation is contributed to the Scientific and Ethics Committee of Tabriz University of Medical Sciences (approval number REC.1396.186.IR.TBZMED) as a thesis for a doctoral degree.

2.2. Inclusion Criteria

Inclusion criteria were the diagnosis of ADHD through psychiatrists' clinical interviews based on indicated criteria in DMS-5 and the age range of 4 to 14 years.

2.3. Exclusion Criteria

Head trauma and epilepsy history, concurrent psychiatric disorder, Intellectual disability, and other severe medical conditions were considered as the exclusion criteria.

2.4. PCR-Gene Amplification

The peripheral blood (in the amount of 3–5 mL) was obtained under sterile conditions and was stored under proper storage circumstances. DNA extraction from blood samples of all participants was performed by the proteinase K method. In the following step, the STin2 (intron2) fragment in the *SLC6A4*

gene was amplified through specific primers, designed using Primer 3 software (version 4), via polymerase chain reaction (PCR), and three types of STin2 alleles of the SLC6A4 gene were examined using the acrylamide gel method. The frequency and distribution of variants were calculated using POPGENE software version 1.32. Then the study's data were coded and analyzed in SPSS software version 26.

2.5 Statistical Analysis

The data were entered, coded, and statistically analyzed in SPSS software (version 26.0; IBM Corp, Armonk, NY, USA), and the mean values and standard deviations were computed with this software. The statistical analysis was performed by Pearson's Chi-square test and Fisher's exact test to compare the frequency of different alleles between the two groups to determine possible associations. The *p-value* < 0.05 was considered statistically significant.

3. Results

A total of 186 children were enrolled in this study. They were divided into two groups, the ADHD group and the control group that contained 86 (46 males and 40 females) and 99 (54 males and 45 females), respectively. All of the participants took part in the study except one of the healthy members who refused the PCR test.

The findings revealed no significant difference between groups in average age (*p-value* = 0.886) and gender (*p-value* = 0.982).

A comparison of the analysis of STin 2 (intron 2) variant alleles, which included 9, 10, and 12 alleles, is as follows.

- Allele 9: The nine positive alleles frequency were compared between two populations as shown in Table 1.

Table 1 Comparison of the frequency of 9 positive alleles between the two groups			
Groups	Frequency	Percentage	<i>p-value</i>
ADHD group	30	34.9	0.527
Control group	39	39.4	
The Odds Ratio for Allele 9 (Positive / Negative) was 0.824 (1.501 – 0.453: 95% CI).			

Table 2
Comparison of the frequency of 10 positive alleles between the two groups

Groups	Frequency	Percentage	<i>p-value</i>
ADHD group	25	29.1	0.366
Control group	23	23.2	
The Odds Ratio for Allele 9 (Positive / Negative) was 1.354 (0.701–2.617: 95% CI).			

Table 3
Comparison of the frequency of 12 positive alleles between the two groups

Groups	Frequency	Percentage	<i>p-value</i>
ADHD group	31	36	0.964
Control group	36	36.4	
The Odds Ratio for Allele 12 (Positive / Negative) was 0.986 (0.541–1.799: 95%CI).			

The Odds Ratio for Allele 9 (Positive / Negative) was 0.824 (1.501 – 0.453: 95% CI).

- Allele 10: The ten positive alleles frequency were compared between two populations as follows.

The Odds Ratio for Allele 9 (Positive / Negative) was 1.354 (0.701–2.617: 95% CI).

- allele 12: The 12 positive alleles frequency were compared between two populations as illustrated in table-3.

The Odds Ratio for Allele 12 (Positive / Negative) was 0.986 (0.541–1.799: 95%CI).

4. Discussion

ADHD is a behavioral disorder in which the neurotransmitters and their balance play a crucial role. As there is a failure in behavioral inhibitions in ADHD, serotonin's role in its pathophysiology has been considered in recent years. It has now been suggested in animal studies that serotonin is involved in hyperactivity, inattention, and impulsive behaviors (5&23). Previous reviews demonstrated the association between the *SLC6A4*, one of the best-studied genes in the psychiatry field, and a wide range of disorders, and we evaluated the relation between the frequency of STin2 variant alleles of this gene and ADHD.

Statistically, none of the STin2 variant alleles had any essential distinction between the two populations in our study.

To assess the risk, the estimated OR values for alleles 9, 10, and 12 were 0.824, 1.354, and 0.986, respectively, and a positive allele 10 had a higher risk of ADHD development.

Banerjee's study in which the polymorphisms of the STin2 variant in patients with ADHD were thoroughly examined, found that the risk of ADHD development had a relation with the inheritance of allele 12 of this variant (24). To Summarize his results and ours, the association of ADHD with the inheritance of allele 12 of this variant can be concluded.

In 2003, Langley, an examiner of *5-HTT* transporter gene polymorphisms, reported no association between the 9, 10, and 12 alleles of the STin2 variant with ADHD (25), which is consistent with our results.

In contrast to our study, Zoroglu in 2002 listed several reasons for the relationship between Homozygosity in Allele 12 (A12 / A12) and an increased risk of ADHD development within the Turkish population, although there was an association with Homozygosity in allele 10 (A10 / A10) and a higher risk of ADHD, similar to our results. (19).

Overall the association between the frequency of STin2 variant alleles of *SLC6A4* and ADHD seems inconsistent. This may be due to the complex genetic architecture of ADHD and the effects of non-genetic factors on the disorder and also the nature of case-control studies evaluating the association. This in turn necessitates more studies with larger sample sizes and case-control studies evaluating maternal and paternal inheritance of variants of genes involved in serotonin homeostasis in patients with ADHD.

Conclusion

In conclusion, there was no association between the frequency of STin2 variant alleles of the *SLC6A4* gene and ADHD, but in the study of risk estimation, it was found that allele 10 of this variant is a risk allele in ADHD patients. However, we recommend further studies with larger sample sizes and among different races in different areas. Also, we suggest case-control studies evaluating maternal and paternal inheritance of variants of genes involved in serotonin homeostasis in patients with ADHD.

Declarations

Ethics approval and consent to participate

This study's confirmation is contributed to the Scientific and Ethics Committee of Tabriz University of Medical Sciences (approval number REC.1396.186.IR.TBZMED) as a thesis for a doctoral degree. Furthermore, Informed consent to participate in this study was obtained from all participant's parent.

Consent for publication

All authors, give their consents for information about this paper to be published in the Journal of Child and Adolescent Psychiatry and Mental Health.

Availability of data and materials

The datasets used and analysed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions:

This work was carried out in collaboration between all authors. Authors SA, MA, NPR, and MSK designed the study and wrote the protocol. This research work was supervised by SA. Authors, SMD, NPR, ARS and MA carried out all practical work. Authors LMF and SA performed the statistical analysis. Author SA, and ARS managed the analyses of the study. Author LMF wrote the first draft of the manuscript. Authors MA, NPR, MSK, and LMF managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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