



Molecular Study 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 one of the most familiar childhood psychiatric disorders. Various molecular genetic reviews indicate that genes are crucial in susceptibility to ADHD. The serotonin transporter gene (*SLC6A4*) has polymorphisms that correlate with ADHD. The association between ADHD and *SLC6A4* gene variants in the Iranian population has not been investigated yet.

Objectives: This study analyzed the STin2 (intron 2) variant of the *SLC6A4* gene in Iranian children and adolescents with ADHD.

Methods: In this retrospective case-control study, 86 ADHD patients and 99 healthy volunteers aged five to 14 were enrolled as the case and control groups, 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 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.

Conclusions: There was no association between the frequency of STin2 variant alleles of the *SLC6A4* gene and ADHD, but in the study of risk estimation, allele 10 of this variant was a risk allele in ADHD patients.

Keywords: Attention-deficit Hyperactivity Disorder; PCR; STin2 Variant; *SLC6A4* Gene

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, which may continue to adulthood in 50 - 80% of cases (1-3). Attention-deficit hyperactivity disorder is described by inattention, hyperactivity, and impulsivity symptoms with a wide range of clinical presentations (4, 5). Considering the importance of ADHD in children, its impact on their health, and its unknown etiology, investigating its genetic factors may help better comprehend, prevent, and possibly cure this disorder.

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 some, including *DRD4*, *DRD5*, *DAT*, *DBH*, *SNAP-25*, *HTR1B*, and *5-HTT*, seem to be statistically involved in the etiology of ADHD (2, 11-14).

The serotonin transporter gene (*SLC6A4*; *5HTT*) is located on chromosome 17q. This gene encodes a carrier protein responsible for retaking serotonin from the synapse and returning it to presynaptic neurons, indicating a crucial role in serotonergic activity regulation within the brain. Attention, memory, and voluntary activity are connected to brain areas such as the amygdala, hippocampus, thalamus, putamen, and anterior cortex, which are the areas expressing *5HTT* (12). The *5HTT* gene could play a significant role in ADHD based on available evidence. 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

among the most studied 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 study showed that STin2.12/STin2.12 homozygotes appear to show fewer serotonin transporters inside the brain (11, 12, 16). In 2002, Zoroglu et al. noted that the STin2.12/12 variant of VNTR polymorphism appears to be associated with an increased risk of ADHD (17).

A study evaluated the ADHD association with two regions' polymorphisms of the 5-HTT gene [variable number of tandem repeats (VNTR) and 5-HTTLRR]. The ADHD group showed a significantly lower 5-HTTLPR S/S genotype but a higher homozygous and heterozygous L variant. Also, the ADHD group indicated a significantly lower VNTR STin2.12/12 genotype, but no significant differences were observed in the frequency of the short (S), long (L), 10, and 12 alleles between the two groups. The study implied that the lack of the S/S variant of 5-HTTLPR polymorphism or the STin2.12/12 variant of VNTR polymorphism is a risk factor for ADHD (17).

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 those without ADHD (18). 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 was observed in short/short 5-HTTLPR genotype in the ADHD type III combined group. Comparing the allele frequencies yielded similar results (19). A review article reported 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).

2. Objectives

Although there are many studies about ADHD, a few have focused on the role of STin2 variants. Therefore, we

aimed to analyze the molecular analysis of the STin2 variants of *SLC6A4* in children and adolescents with ADHD.

3. Methods

3.1. Sample

The study group comprised 86 children from Northwest Iran diagnosed with ADHD based on the Diagnostic and Statistical Manual (DSM-5) criteria (20). The control group consisted of 99 non-psychiatric participants with similar demographic features such as age and gender who were referred to a children's hospital affiliated with Tabriz University of Medical Sciences for adenotonsillectomy and routine lab tests. We followed a convenience sampling technique performed by a psychiatrist based on inclusion and exclusion criteria. Additionally, participants' parents filled out informed consent forms. This study was confirmed by 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.

3.2. Inclusion Criteria

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

3.3. Exclusion Criteria

The exclusion criteria were head trauma and epilepsy history, concurrent psychiatric disorder, intellectual disability, and other severe medical conditions.

3.4. PCR Gene Amplification

The peripheral blood (3 - 5 mL) was obtained under sterile conditions and stored under proper storage circumstances. The DNA was extracted from the blood of all participants per the proteinase K method. Next, 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). 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 version 1.32 software.

3.5. 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.

4. Results

A total of 186 children were enrolled in this study. They were divided into two groups, the ADHD group and the control group, which contained 86 (46 males and 40 females) and 99 (54 males and 45 females) people, respectively. However, one of the healthy participants refused the PCR test and was excluded.

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

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

- Allele 9: The allele 9 frequencies were compared between the two groups, as shown in [Table 1](#).

Table 1. Comparison of the Frequency of Nine Positive Alleles Between the Two Groups

	Frequency (%)	P-Value
Groups		0.527
ADHD group	30 (34.9)	
Control group	39 (39.4)	

The odds ratio for allele 9 (positive/negative) was 0.824 (95% CI: 0.453 - 1.501).

- Allele 10: The allele 10 frequencies were compared between the two groups, as presented in [Table 2](#).

Table 2. Comparison of the Frequency of 10 Positive Alleles Between the Two Groups

	Frequency (%)	P-Value
Groups		0.366
ADHD group	25 (29.1)	
Control group	23 (23.2)	

The odds ratio for allele 10 (positive/negative) was 1.354 (95% CI: 0.701 - 2.617).

- Allele 12: The allele 12 frequencies were compared between the two groups, as illustrated in [Table 3](#).

The odds ratio for allele 12 (positive/negative) was 0.986 (95%CI: 0.541 - 1.799).

Table 3. Comparison of the Frequency of 12 Positive Alleles Between the Two Groups

	Frequency (%)	P-Value
Groups		0.964
ADHD group	31 (36)	
Control group	36 (36.4)	

5. Discussion

Attention-deficit hyperactivity disorder is a behavioral disorder in which 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, 21). Previous reviews demonstrated the association between *SLC6A4*, one of the best-studied genes in the psychiatry field, and a wide range of disorders, and we evaluated the relationship 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 allele 10 increased the risk of ADHD. Banerjee's study thoroughly examined the polymorphisms of the STin2 variant in patients with ADHD and found that the risk of ADHD was related to the inheritance of allele 12 of this variant (22). 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 alleles 9, 10, and 12 of the STin2 variant and ADHD (23), 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 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 (17).

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, the effects of non-genetic factors on the disorder, and 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.

5.1. Conclusions

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, allele 10 of this variant was 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.

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Footnotes

Authors' Contribution: SA, ASK, & MSK contributed to the study design and lab experiments. MA, SMD, NPR, and LMF performed all lab work, statistical analyses, and interpretation of data. SM and MSK supervised the study and contributed to all parts of the paper. Intellectual content was revised and approved by SA, MSK, and LMF. LMF wrote the paper, and all authors read and approved the final version of the manuscript.

Conflict of Interests: All authors confirm no competing interest.

Ethical Approval: This study was approved by 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.

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Informed Consent: Participants' parents filled out informed consent forms.

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