ORIGINAL ARTICLE

Study of Changes in Rs2283265 Polymorphisms in Dopamine Receptor D2 and Rs27072 in Dopamine Transporter Gene (SLC6A3) in Patients with Attention-deficit Hyperactivity Disorder

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Abstract

Objectives

Attention-deficit hyperactivity disorder (ADHD) is one of the most common psychiatric disorders in children that lead to numerous complications. This study examined the changes in rs2283265 polymorphisms in the dopamine receptor D2 (DRD2) and rs27072 in the dopamine transporter gene (SLC6A3) in ADHD patients.

Materials & Methods

This descriptive-analytical study was performed on children aged 4-12 years with ADHD. In this study, 100 patients in the ADHD group (according to DSM-IV-TR criteria and diagnosed by interview by a child and adolescent psychiatrist) and 100 children in the control group (including patients referring to the pediatrician without hyperactivity) were enrolled.

Two polymorphisms rs2283265 and rs27072 in two groups were comparatively investigated using PCR-RFLP method and restriction enzymes. Data were analyzed using SPSS 17.

Results

There was a significant correlation between gender and ADHD, and the disease was more common in boys (P=0.021).

In this study, there was no significant relationship between ADHD types and frequency distribution of rs2283265 (DRD2) and rs27072 (SLC6A3) polymorphism genotypes (P<0.05). However, there was a significant correlation between distribution of rs2283265 (DRD2) and rs27072 (SLC6A3) polymorphisms and ADHD (P<0.05).

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Conclusion

It seems that the changes in DRD2 and SLC6A3 genes are associated with ADHD, and study of these genes can be helpful in diagnosis and genetic screening.

Keywords: Attention-deficit hyperactivity disorder; Behavior disorder; Gene, Dopamine
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Introduction

Attention-deficit hyperactivity disorder (ADHD) is one of the most important causes of referral of children to clinics across the world and one of the common psychiatric disorders in Iran (1).

This disease leads to certain disorders such as hyperactivity, distractibility and inattention in children. ADHD makes the patients predisposed to unhealthy lifestyle and health problems (2), and leads to educational decline and certain problems in the social relationships in affected children (3).

Numerous prediaposing factors such as substance abuse, exposure to heavy metals and chemicals, and unhealthy lifestyle during pregnancy are thought to contribute to the development of disease (4).

But genetic factors are one of the most important factors in the development of this disease. For example, the presence of LPHN3 and CDH13 genes in these patients is one of the causes of the disease (5). But the nature of the disease is complex and polygenic, and it is therefore essential to examine all the genetic aspects of the disease (6).

Increased expression of the genes involved in the onset of the disease affects the brain and/or its receptors and causes the symptoms of the disease to develop. In this regard, neurotransmitters, including dopamine, play an important role in regulating memory, attention, reward, emotional states and human stimulation (7, 8).

Therefore, hyperactivity stimulant drugs increase dopamine by strengthening weak dopamine signals in the brain (9, 10).

Specifically, in hyperactivity patients, there is a particular variety of genes known as dopamine transporter gene (SLC6A3) and dopamine receptor D2 (DRD2) (11).

Polymorphism rs2283265 is located in intron 5 of the DRD2 gene. This polymorphism causes a change in gene expression by affecting mRNA splicing, and consequently learning and attention control are influenced (12).

In addition, rs27072 polymorphism is located at 422 bp in the upstream VNTR region in the 3'UTR of the dopamine transporter gene, namely, SLC6A3. This change can affect the function of this transporter by decreasing the protein levels (13).

Since genetic factors along with environmental factors are among the main causes of ADHD (14), it is therefore essential to conduct genetic studies on the genes encoding the products involved in the production of dopamine and the study of polymorphisms involved in the incidence of ADHD.

In this study, we investigated the changes in rs2283265 polymorphism in the DRD2 and

rs27072 gene in the SLC6A3 gene in ADHD patients referring to Shahrekord Imam Ali (AS) Clinic.

Materials & Methods

This cross-sectional study was conducted on children aged 4-12 years with ADHD who referred to the Psychiatric Clinic of Imam Ali Hospital in Shahrekord in 2017-2018.

Sampling was conducted by probability (convenience, random) method. A total of 200 people, consisting of 100 children with ADHD in the case group and 100 subjects in the control group (including patients referring to the pediatrician without ADHD and matched with the case group) were studied. The number of individuals in each group was estimated according to a sample size calculation formula and a similar study (15).

The inclusion criteria included ADHD patients based on DSM-V diagnostic criteria aged 4-12 years (16), lack of other medical conditions, and lack of drug use. Lack of collaborating with tests and research procedure, death, or loss of samples during the study were considered exclusion criterion.

Peripheral blood samples were taken and kept at -20 °C in the freezer until molecular tests were performed.

Peripheral blood samples were taken with EDTA anticoagulant and total cell DNA extraction was conducted by using Sinaclon kit (Iran).

To obtain a better quality DNA and higher purity, the red blood cells were first lysed with a lysis buffer, and then the total cell DNA was extracted from the resulting buffy coat.

To determine the concentration of the extracted DNA and investigate its contamination, a Nanodrape device (TermofisherNono drop 2000) was used, and to determine the rate of absorbance and contamination with phenolic compounds, optical absorbance at 260 nm (absorbance rate of nucleic acids), 280 nm (absorbance rate of protein), and 230 nm (absorbance rate of phenolic compounds) was used.

The sequences of DRD2 and SLC6A3 genes were obtained from the NCBI database.

Appropriate primers were designed using the oligo7 software, analyzed for specificity and efficiency, and blasted by using the NCBI database.

For the DRD2 gene, due to the presence of mismatch created in the F2 primer sequence and its lack of precise and specific binding to the other two external primer genomes, nested PCR was conducted.

The sequences of primers R1 and R2 overlap. The sequence of primers and the characteristics of the restriction enzymes are shown in Table 1.

For amplification of DNA strands, polymerase chain reaction (PCR) on all of the samples was performed on rs2283265 polymorphisms in the DRD2 gene and the rs27072 gene in the SLC6A3 receptor gene.

Table 2 showed that lists of the amounts of compounds used in PCRs and their temperature protocols in thermocycler.

Electrophoresis of PCR products was performed on 8% polyacrylamide gel and staining was conducted by using silver nitrate.

The genotypes were examined and the polymorphisms were detected using PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) by using using the AluI restriction enzyme for rs2283265 polymorphism and the MspI enzyme for SLC6A3 polymorphism.

Samples were stored in incubator for 16 hours at 37 °C. PCR-RFLP products were evaluated

to determine the size of the resulting digested fragments and were electrophoresed on polyacrylamide gel, and after staining the gel, the presence of polymorphism was investigated.

Data from laboratory tests were recorded in the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS).

In a study conducted in Iran, its reliability was obtained 0.81, 0.67, and 0.56 for ADHD, oppositional defiant disorder (ODD), and tic disorder, respectively, by Cronbach's alpha coefficient, and it was also found to be highly reliable (17).

Verbal and written consent was obtained from parents and all the ethical standards of the Helsinki Declaration were observed in the study protocol.

Finally, the data were entered into the SPSS version 22. Data analysis was performed using chi-square test, independent t-test, ANOVA, and paired t-test to compare the mean post-intervention values of quantitative variables after adjustment of baseline values.

$P < 0.05$ was considered significance level.

Results

After performing PCR-RFLP on the samples in the two groups, PCR products were first isolated on 8% polyacrylamide gel to investigate whether the PCR was optimal or not.

As seen, single-band products were formed in the place of interest. After enzyme digestion on PCR products for each polymorphism, the sequences were re-isolated on 8% polyacrylamide gel. The results of enzyme digestion for the two polymorphisms are illustrated in Figures 1 and 2.

In this study, the following results regarding the changes in rs2283265 polymorphisms in the DRD2 and rs27072 in the SLC6A3 gene in ADHD patients were obtained

A total of 200 children participated in this study who were assigned to two groups of 100 each, namely, patients and controls. The age of children was 4-12 years.

In the case group, 77 cases were male, 23 were female, and control group consisted of 75 boys and 25 girls. Given the significance level of 0.021, a significant relationship between gender and incidence of ADHD was observed.

The results regarding the frequency

Given the significance level, a relationship was observed between the frequency distribution of rs2283265 polymorphism (DRD2) and group membership such that the frequency of TT homozygous genotype was 1% in the patient group, While the frequency of TT homozygous genotype was 6% and also the frequency of GG homozygous genotype was 97% in the control group, while in the control group, the frequency of homozygous GG genotype was 85%.

The heterozygous TG genotype frequency was 2% in the patients group and 9% in the control group. It should be noted that the genotypic distribution of the studied polymorphisms follows the Hardy-Weinberg equilibrium in the two groups.

The results regarding the frequency of T and G alleles of the rs2283265 (DRD2) polymorphism in the two groups of patients and controls are presented in Table 5.

Given the significance level, there was a relationship between the frequency distribution of T and G alleles of the rs2283265 (DRD2) gene and group membership, so that the frequency of T allele in the patient group was 2%, while in the control group, it was 10.5%.

The results regarding the frequency of the rs27072 polymorphism genotypes in the SLC6A3 gene in

patient and control groups are presented in Table 6. Given the significance level, a significant relationship was noted between the distribution frequency of the polymorphism rs27072 (SLC6A3) and group membership, so that the frequency of TT homozygous genotype in the patient group was 1%, while in the control group, the frequency of this genotype was 5% and in the patients group, the frequency of homozygous GG genotype was 97%, while in the control group, the frequency of this genotype was 85%.

In addition, the frequency of TG heterozygous genotype was 2% in the patients group and 10% in the control group.

It should be noted that the genotypes distribution of the studied polymorphisms follows the Hardy-Weinberg equilibrium in both groups.

The results on the frequency of T and G alleles of the rs27072 (SLC6A3) polymorphism in patient and control groups are presented in Table 7.

Given the significance level, there was a relationship between the frequency distribution of T and G alleles of rs27072 (SLC6A3) and group membership, so that the frequency of T allele in the patient group was 2% while in the control group, it was 10%.

Table 1. The characteristics of primers used to study polymorphisms rs27072 and rs2283265

Gene	Polymorphism	Primer sequence	Digestion site	Product length (bp)
DRD2	rs2283265	F1: GATGTCTTGTGAATCTCCCCTTCACTC R1:AGGAACACAAGGACCTGGTTTTTTATGG F2: GCTGAGTGACCTTAGGCAAGTA R2:TGGGTATGACGAGGAACACAAG	AG,CT	310 TT: 197 TG: 174+23+ 197 GG: 174+23
SLC6A3	rs27072	F1: CGCCGTGTCTTGTGTTGCT R1:ACGGGGATTCTCAGCAGGTG	C,CGG	AA: 219 AG: 137+82+ 219 GG:137+82

Table 2. The amounts of compounds used in the PCR1 and PCR2 solutions for the DRD2 gene and in the PCR solution for the SLC6A3 PCR gene

Compounds	Concentration (of all three genes)	Volume (microL)		
		DRD2 gene		PCR SLC6A3 gene
		PCR1	PCR2	
H2O	-	4/3	8	4/8
Master Mix	-	5	10	10
Primer (Forward, Reverse)	pM/ µL 10	6/0	1	6/0
DNA	ng/**µl** 20-50	1	1	1
Total volume	-	10	20	20

Table 3. The temperature protocols of polymerase chain reactions for studied genes

PCR cycle	PCR1 gene DRD2		PCR2 gene DRD2		PCR gene SLC6A3		Cycle no.
	Temperature (ºC)	Temperature	Temperature (ºC)	Temperature	Temperature (ºC)	Temperature	
Denaturation	95	5 min.	95	5 min.	95	5 min.	1
Annealing	95	40 seconds	95	30 seconds	95	35 seconds	30
	61	40 seconds	57	30 seconds	60	35 seconds	
	72	40 seconds	72	30 seconds	72	35 seconds	

Table 4. Frequency distribution of rs2283265 (DRD2) polymorphism genotypes in children in patient and control groups

	Genotype Frequency (percentage)	TT		GG		TG		Total		P-value*
		Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Groups	Patient group	1	1	97	97	2	2	100	100	012/0
	Control group	6	6	85	85	9	9	100	100	
	Total	7	5/3	182	91	11	5/5	200	100	

* Fisher's exact test

Table 5. Frequency distribution of T and G alleles of rs2283265 (DRD2) polymorphism in children of two groups

	Genotype Frequency (percentage)	T		G		Total		P-value*
		Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Groups	Patient group	4	2	196	98	200	100	021/0
	Control group	21	5/10	179	5/89	200	100	
	Total	25	25/6	175	78/93	400	100	

* Fisher's exact test

Table 6. Frequency distribution of rs27072 (SLC6A3) polymorphism genotypes in children in patient and control groups

	Genotype Frequency (percentage)	TT		GG		TG		Total		P-value*
		Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Groups	Patient group	1	1	97	97	2	2	100	100	01/0
	Control group	5	5	85	85	10	10	100	100	
	Total	6	3	182	91	12	6	200	100	

* Fisher's exact test

Table 7. Frequency distribution of T and G alleles of the rs27072 (SLC6A3) polymorphism in children of patient and control groups

	Genotype Frequency (percentage)	T		G		Total		P-value*
		Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Groups	Patient group	4	2	196	98	200	100	017/0
	Control group	20	10	180	90	200	100	
	Total	24	6	376	94	400	100	

* Fisher's exact test

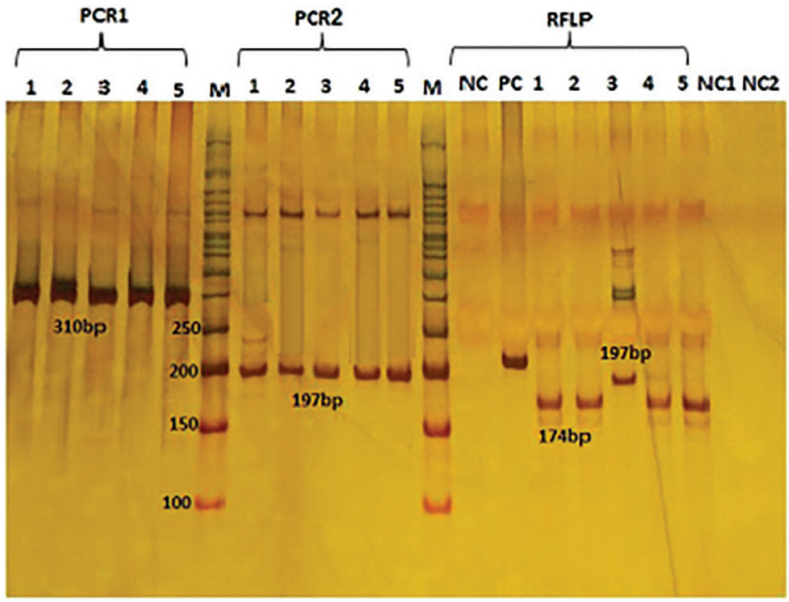

Figure 1. *PCR and RFLP-PCR products for rs2283265 polymorphism in D2 receptor dopamine receptor gene (DRD2) in optimal conditions on 8% polyacrylamide gel for sequencing. M: Marker 50bp, NC: Negative Control, PC: Enzyme-free Control, in RFLP: No. 1, 2, 4, and 5 Genotype GG, No. 3 TT Genotype.*

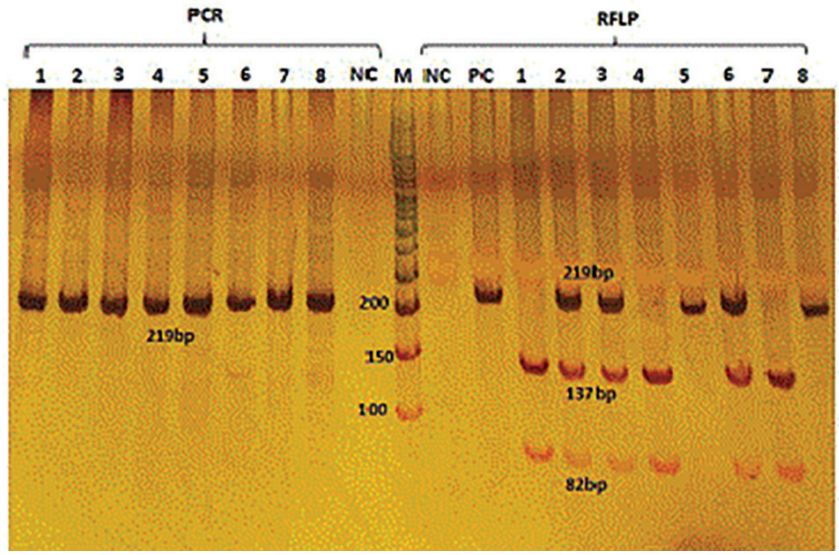

Figure 2. *PCR and RFLP-PCR products for rs27072 polymorphism in the dopamine transporter gene (SLC6A3) under optimal conditions on 8% polyacrylamide gel for sequencing. M: Marker 50bp, NC:Negative control, PC: Enzyme-free control, in RFLP section: No. 1, 4 and 7 Genotype GG, No. 2, 3 and 6 Genotype AG, No. 5 and 8: Genotype AA.*

Discussion

In this study, the frequency of two polymorphisms rs2283265 in D2 receptor dopamine receptor (DRD2) and rs27072 in the dopamine vector gene (SLC6A3) in children with ADHD was studied.

In the present study, given the significance level, a significant relationship was noted between the frequency distribution of rs2283265 polymorphism genotypes in DRD2 gene and ADHD in regard to the significance.

In addition, there was a significant relationship between the frequency of T and G alleles of the rs2283265 polymorphism in the DRD2 gene.

Consistent with the results of this study, a study on children with ADHD showed that there was a significant difference in genotypes and alleles frequency of the DRD2 polymorphism between healthy and patient groups, and this gene was involved in the pathogenesis of disease (18).

Gadow et al. showed that DRD2 rs2283265 was effective in treating inattention to teacher behavior, and the dopaminergic gene polymorphisms played a role in modulating behavior in ADHD and in emotion dysregulation (19).

Some studies have also shown that the density of DRD2 is associated with increased task-switching efforts and is effective in creating new and innovative behaviors (20).

Another study showed that two polymorphisms rs1800497 and rs2283265 near the DRD2 gene could enhance learning and cognitive activity (21).

But some studies have reported inconsistent results with this study. For example, a study showed that the role of DRD2 TaqI in development of ADHD symptoms in childhood was questionable, and more studies are needed (22).

Another study also found that there was no significant relationship between DRD2 gene (-141C Ins/Del, rs1799732) and ADHD in a children population of northeastern Iranian (23).

Kirley et al. also studied that two polymorphisms of the DRD2 gene in 118 ADHD children and their families, and reported no significant relationship (24).

DRD1 and DRD2 polymorphisms were associated with stability of attention problems in adolescence,

youth, and adulthood, but this association was not found in childhood (25).

Therefore, the difference in demographic characteristics such as age may be a determinant factor in cognitive activities, attention and concentration of individuals, which could explain the inconsistency in the findings of different studies.

In this study, a significant difference was seen in the frequency distribution of rs27072 polymorphism (SLC6A3) between the two groups, and therefore a relationship between this polymorphism and ADHD disease exists.

There was also a significant relationship between the frequency of T and G alleles of the rs27072 (SLC6A3) polymorphism in DAT1 gene.

Similarly, a review article has reported that SLC6A3 and DRD4 genes are one of the most important recurring causes of psychological disorders in ADHD that can contribute to the pathogenicity of the disease by interfering with the brain dopamine system (26).

Most studies on the relationship between ADHD and DAT1 have examined VNTR polymorphisms in the '3 UTR of the DAT1 gene. In a study, the association of polymorphism (rs2975226) T/67A- and (rs2652511) 839C/T in the DTA1 gene promoter region was studied in a sample of Ukrainian and Taiwanese ADHD patients.

The results showed that there was a significant association between T-allelic polymorphism -67A/T promoter and ADHD in Taiwan, but no significant correlation was found between polymorphism -839C/T and ADHD in the two populations.

Generally, the results of this study showed that the genetic diversity of the DAT1 promoter region could be considered a risk factor for ADHD disease progression (27).

Jeong et al. examined the ADHD characteristics of adults, and found that DAT1 gene polymorphisms (rs27072, rs11133767, rs429699, rs27048, rs2937639) played a role in the mood instability in adults (28).

Another study showed that there was no significant relationship between 40-bp dopamine transporter gene polymorphism and ADHD in the Iranian population, and suggested that considering the key role of dopamine transporter in ADHD autopathophysiology, further analysis of other functional SNPs in this gene in ADHD patients is needed (29).

In the studies Gadow et al. and Feng et al., several genotypes in childhood were found to be involved in the incidence of ADHD, one of which is DAT1 rs27072 (C-C vs. T-C), which was sequentially observed (30, 31).

However, despite these results, some studies reported that the locus of SLC6A3 was not effective in responding to methylphenidate in ADHD patients, and more studies in this regard are required (32).

In general, studies have shown that DRD2 and SLC6A3 genes interact with each other, and in the same way, their polymorphisms also affect each other's expression (33, 34).

As a result, changes in the level of neurotransmitters such as dopamine are effective in the development of abnormal behaviors due to ADHD.

In Conclusion

There is a relationship between the distribution of rs2283265 polymorphism in the DRD2 gene and the distribution of rs2283265 polymorphism genotypes in this gene and ADHD types.

This polymorphism causes a change in the gene expression and possibly causes lower ADHD

symptoms by producing a lower protein content by affecting the mRNA splicing.

It is recommended in future studies to study the association of the studied polymorphisms or other polymorphisms with other genes and variants in the DRD2 and SLC6A3 genes to provide more accurate associations between genetic factors and the incidence of ADHD.

Author's Contribution

Abolfazl Khoshdel contributed to the study conception and design. Parvin Safavi contributed in case selection and and led monitoring and implementation of the study. Laboratory practice was performed by Effat Farrokhi, Hossein Soleimani Farsani, and Nika Khoshdel. Afsaneh Malekpoor performed statistical analysis. All authors cooperated in executive steps of the study. The first draft of the manuscript was written by Abolfazl khoshdel, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Conflict of interest

The authors of this article have no conflicts of interest.

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