J Compr Ped. 2021 November; 12(4):e109377. doi: 10.5812/compreped.109377.

Published online 2021 December 4. **Research Article**

Analysis of Association Between the Effects of Methylphenidate and *DRD4* Gene Polymorphisms in Patients with Attention Deficit Hyperactivity Disorder

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Received 2020 October 27; **Revised** 2021 October 10; **Accepted** 2021 October 19.

Abstract

Background: Drug treatment is one of the most important treatments for attention deficit hyperactivity disorder (ADHD). The *DRD4* gene is a transporter and receptor coding gene of dopamine and is one of the most important genes under investigation in the disorder and etiology of ADHD. In this study, the association between *rs3758653* C/T and VNTR exon 3 repetition polymorphisms of the *DRD4* gene and the effects of methylphenidate were investigated in patients with ADHD disorder consuming methylphenidate.
Methods: The descriptive-analytical study was performed on 122 patients (5 - 18 years old) with ADHD who were treated with methylphenidate. DNA was extracted using salting out method. Subsequently, the *rs3758653* polymorphism in the 5'UTR region of *DRD4* gene was genotyped by PCR-RFLP method, and the VNTR fragment in exon III of *DRD4* gene was investigated by electrophoresis gel on acrylamide gel method. After eight weeks from the start of drug treatment with methylphenidate, the intensity of symptoms was evaluated using the Conners scale. Finally, all data from questionnaires and information that were resulted from laboratory findings were analyzed using ANOVA and repeated measure analysis.
Results: Of the 122 patients under study, 15 patients (12.3%) were responded to the drug treatment, and 107 patients (87.7%) were not responded. The significant differences were not revealed in genotype, and allele frequencies of between *rs3758653* (C/T) and exon III 3'VNTR repeats polymorphisms of the *DRD4* gene and responder and non-responder of ADHD groups to the drug treatment.
Conclusions: The results showed that the reduction of ADHD symptoms with drug treatment is not related to *DRD4* sub-types in patients with ADHD.

Keywords: ADHD, *DRD4*, Methylphenidate

1. Background

Attention deficit hyperactivity disorder (ADHD) is one of the most common psychiatric problems in school age children (1). ADHD is a complex and heterogeneous disorder with unknown etiology (2, 3). In other words, ADHD is considered a multifactorial disorder, and its contributing factors include psychological, environmental, and genetic factors (4). Many studies suggest that an important factor of the pathogenesis of hyperactivity disorder may be a genetic factor. Also, data obtained from families and twins indicate that ADHD has genetic background, and 80% of genetic factors are involved in the phenotype of the disease (5).

Drug treatment is one of the most important treatments for ADHD. Drugs used as primary treatment for ADHD affect the dopaminergic system, so transporter and dopamine receptor coding genes are the most important genes under study in this disorder (6). The psychostimulant methylphenidate is the most frequently used medication to treat ADHD. Several studies have investigated the benefits of methylphenidate, showing possible favorable effects on ADHD symptoms. Therefore, the genes related to this system have been really considered in different studies (7).

Dopamine receptor D4 (*DRD4*) is one of the places of interest for investigating and studying the etiology of ADHD. The gene is located on chromosome 11p15.5 (8). The most widely studied gene in the dopaminergic pathway is the dopamine receptor D4, which is encoded by the *DRD4* gene; among the most explored genetic variables are the variable number tandem repeats (VNTR), located in exon III of this gene. However, studies that evaluated single nucleotide polymorphisms (SNPs) are scarce. In addition to this SNP, one SNP is located in the promoter region (*rs3758653*).

DRD4 gene that is expressed in several brain areas has also been investigated in relation to ADHD (9). Linkage mapping shows the locus of the gene on chromosome 11p15.5 (10). This gene has several polymorphisms in its nucleotide sequence. The 48-base pair variable number tandem repeats (VNTR) in exon III of *DRD4* polymorphism is the most studied polymorphism in association with ADHD. Biological molecular studies show that this region couples with G-protein, modulating cAMP production (Van Tol & Guan, 1992). VNTRs are repeat sequences with varying lengths and can have 2 - 11 repeats, but their common variants are 7, 4, 2 (11). Recent studies have shown that the 7-repeat variant has a weaker response to dopamine than other variants (11). Therefore, there is a positive relationship between the 7-repeat allele and ADHD disorder, but some studies do not show a relationship between them.

The cause of this inconsistency in the results is not well established yet. It has not been determined whether the differences in reported results have been due to differences in sampling groups, genetics, and heterogeneity, or weaknesses in the interpretation of the statistical tests, or indeed represent a real difference between different populations.

2. Objectives

Because of the contradictory results, the current study was performed to examine the potential role of *DRD4* gene polymorphisms in ADHD patients using methylphenidate for the treatment.

3. Methods

The descriptive-analytical study was performed on ADHD patients, aged 5 - 18 years, in Tabriz, Iran during 2016, who were referred to child and adolescent psychiatry clinic in this city for receiving psychiatric services and needed drug treatment.

The sampling was accomplished based on convenience method with regards to the inclusion and exclusion criteria. Patients with ADHD were included in the study based on criteria specified in the DSM-IV-TR, by getting interviewed by a child and adolescent psychiatrist, and semi-structured interview form of PL-SADS-K in the age range of 5 to 18 years after parental consent. In addition, a semi-structured K-SADS diagnostic questionnaire designed according to R-III-DSM and IV-DSM criteria were completed by a psychiatrist through interviews with parents and participants. Finally, patients with a history of head trauma, major psychiatric simultaneity disorder, epilepsy, serious medical illness, and mental retardation were excluded. Eventually, a group of children ($n = 122$) with ADHD disorder was selected.

The reliability of the Persian version of this test with the retest method studied by Ghanizadeh et al. was reported 0.81 and 0.69 inter-rater (12). Also, in this study, the ADHD rating scale questionnaire and parental form were used to differentiate ADHD children from the clinical control group, attention deficit symptoms of hyperactivity and impulsivity symptoms, and to differentiate different types of ADHD. The validity and reliability of the questionnaire were reported above 0.75 (13).

After completing the questionnaires by the parents and the patients, 4 mL of peripheral blood were obtained from each child, and DNA extraction was performed using salting out method and subsequently used as a template for determination of *DRD4* gene genotypes. Genotyping *rs3758653* polymorphism in the 5'UTR region of *DRD4* gene was investigated by using PCR-RFLP method. PCR products were digested with Ecor I restrictive enzyme (Table 1).

The polymorphism of VNTR fragment in exon III of *DRD4* gene was investigated by electrophoresis gel on acrylamide gel method. In this method, the genotype of each individual is obtained by comparing the size of the DNA marker, which is loaded on the gel with the sample. The size of the bands will also change as the number of repeats from person to person.

All of the ADHD subjects were administered methylphenidate (Ritalin®) for eight weeks. The dosages were enhanced up to a sufficient dose to accomplish the therapeutic effect, established by the parents' reports of symptom improvement and side effects. Subsequently, these dosage levels were maintained for eight weeks. Eight weeks after the start of drug treatment with methylphenidate (Ritalin®), the intensity of symptoms was evaluated using the Conners scale for the second time. Response to the treatment was defined as at the least 50% decrease in Conners score (14). Finally, all data from questionnaires and information that were resulted from laboratory findings were analyzed using ANOVA and repeated measure analysis.

Table 1. Primers Sequences of *rs3758653* Polymorphism and VDTR Exon 3 of *DRD4* Gene

Primer Sequencing (5'→3')		Product Size
rs3758653		264bp
F	AGAGTGGTGCCCCCTTTTAG	
R	CAAGACCGTGAGCTAGGTAGG	
Exon III VNTR		One repeat: 600 bp; two repeat: 700 bp; four repeat: 800 bp; five repeat: 850 bp; six repeat: 900 bp
F	CGTACTGTGCGGCCTCAACGA	
R	GACACAGCGCCTGCGTGATGT	

4. Results

The study was implemented on 122 patients with ADHD disorder (Table 2). The ADHD rating scale questionnaire was evaluated before starting the study, and its mean score in patients was obtained 28.5 ± 11.96. The mean intensity of symptoms was calculated as 44.98 ± 16.99 in investigating patients with Conners questionnaire.

Table 2. Demographic Characteristics of Patients with ADHD [a]

Variables	Values
Gender	
Male	118 (15.7)
Female	22 (84.3)
Age	9.05 ± 2.88
Weight	31.6 ± 12.9
Conner's rating scale	44.98 ± 16.9
Psychiatric comorbidity	12 (8.6)
Major depressive disorder	1 (0.7)
Obsessive compulsive disorder	1 (0.7)
Tic disorder	2 (1.4)
Anxiety disorder	3 (2.1)
Oppositional defiant disorder	5 (3.6)

[a] Values are expressed as No. (%) or mean ± SD.

In *rs3758653* polymorphism, three genotypes of TT (normal), TC (heterozygote), and CC (mutant) can be identified in different individuals. Genotypes of TT were homozygous dominant, while CC were homozygous recessive. The frequency of CC homozygous recessive in ADHD patients was 16 children (13.11%). Also, the frequency of the most common form of the exon III VNTR polymorphism (2705 bp repetition) was observed in 93 children (76.22%) (Tables 3 and 4).

No significant relationship was observed between alleles and genotype frequencies of *rs3758653* polymorphism and exon III VNTR with responding to drug therapy among responder and non-responder ADHD groups.

Among 122 ADHD children, 107 (87.7%) patients did not respond appropriately to drug treatment (non-responder group), and 15 (12.3%) patients were responded appropriately (responder). Among the non-responder group, 51 (41.8%) patients showed CT genotype of *rs3758653* polymorphism, and 83 (67.8%) patients showed two repeat alleles of exon III VNTR (Figures 1 and 2).

5. Discussion

Early treatment of ADHD can prevent many of its complications. Because of reasons which have not been determined yet, the response to treatment is not the same in patients with ADHD. In this regard, D4 receptors regulate a number of signaling events, including adenylate cyclase inhibition, stimulation of arachidonic acid release, and modification of potassium channels. The *DRD4* gene contains four exons and encodes a 387-amino acid protein. The gene is highly expressed in pyramidal, frontal cortex, and retinal neurons, but the intensity of its expression is low in basal ganglia, hippocampus, and thalamus. The relationship between the types of genetic variants in *DRD4* sequences has been investigated along with a variety of neurological diseases, and its relationship with ADHD disease has been confirmed in some studies but rejected in some others (13). Also, some studies have investigated the effect of different polymorphisms of the gene and its response to the drug treatment that has been differently implemented in different populations (15).

According to the results of the present study and compared to ADHD groups (responder and non-responder), no significant relationship was observed between genotype and allele frequencies of exon III VNTR and *rs3758653* polymorphisms with responding to drug treatment in ADHD groups.

Also, in the non-responder group, 107 patients did not respond appropriately to drug treatment which among these, 51 (41.8%) patients showed CT genotype of *rs3758653* polymorphism, and 83 (67.8%) patients showed 2R repeat allele of exon III VNTR. The total results of this study

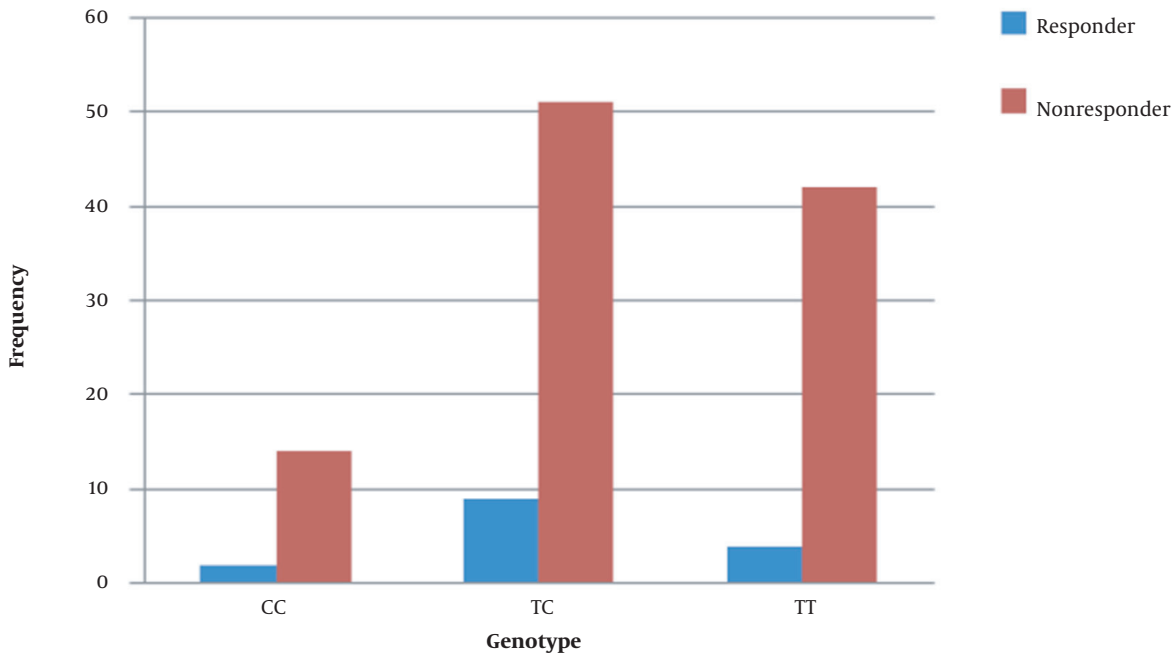

Figure 1. Genotype frequencies for *DRD4rs3758653* polymorphism in children with ADHD regarding response to treatment with methylphenidate.

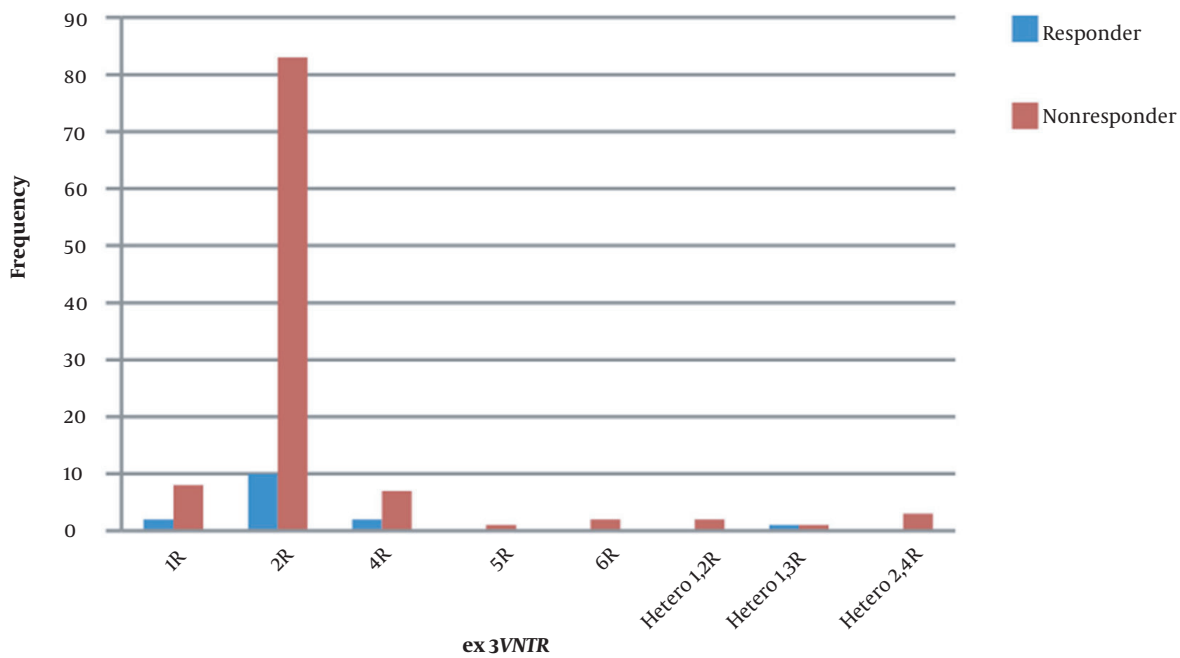

Figure 2. Allele frequencies for exon III VNTR of *DRD4* gene in children with ADHD regarding response to treatment with methylphenidate.

Table 3. Allele and Genotype Frequencies of *rs3758653* Polymorphism of *DRD4* Gene Among Responder ADHD Patients in Comparison to Non-responder ADHD Patients to Methylphenidate Treatment [a]

Genotype/Allele (rs3758653)	Responder (n = 15)	Non-responder (n = 107)	OR (95%CI)	P-Value
CC	2 (1.6)	14 (14)	0.646 (0.064 - 4.553)	0.634
CT	9 (7.4)	51 (41.8)	1 (reference)	
TT	4 (3.3)	42 (34.4)	0.109 (0.576 - 0.602)	0.377
C	13 (4.33)	79 (36.91)	1.307 (0.293 - 5.672)	0.689
T	17 (5.66)	135 (63.08)	1 (reference)	

[a] Values are expressed as No. (%).

Table 4. Allele Frequencies of Exon III VNTR of DRD4 Gene Among Responder ADHD Patients in Comparison to Non-responder ADHD Patients to Methylphenidate Treatment [a]

Genotype/Allele (Exon III VNTR)	Responder (n = 15)	Non-responder (n = 107)	OR (95%CI)	P-Value
1R	2 (1.7)	8 (6.6)	2.104 (0.211 - 19.923)	0.418
2R	10 (8.3)	83 (67.8)	1 (reference)	
4R	2 (1.7)	7 (5.8)	2.394 (0.235 - 18.865)	0.344
5R	0 (0)	1 (0.8)	0.00 (0.00 - 340.157)	0.754
6R	0 (0)	2 (1.7)	0.00 (0.00 - 52.089)	0.649
Hetero 1,2R	0 (0)	2 (1.7)	0.00 (0.00 - 52.089)	0.649
Hetero 1,3R	1 (0.8)	1 (1.6)	4.084 (0.064 - 95.845)	0.285
Hetero 2,4R	0 (0)	3 (1.6)	0.00 (0.00 - 57.0804)	0.658

[a] Values are expressed as No. (%).

showed that the reduction of ADHD symptoms with drug treatment is not related to *DRD4* sub-types in patients with ADHD.

In agreement with the results of our study, a study performed in the Korean population did not show a relationship between polymorphism of *DRD4* gene and the response to treatment with methylphenidate (14). Also, Brookes et al. reported that the *DRD4* gene in ADHD patients in Taiwan is not related to the incidence or intensity of this disease, and the incidence of this allele is approximately the same in patients with ADHD and control group (16).

Qian et al. performed two studies in China and did not observe a significant association between four and six repeats of VNTR alleles and the incidence of ADHD (17, 18). These findings were confirmed by Leung et al. (19), who investigated the repeat II VNTR alleles in patients with ADHD. Carrasco et al., in 2006, in a Chilean population, also reported a negative relationship between 7-repeats and ADHD (20). However, contradictory findings have been reported in many studies. Tabatabaei et al. revealed two to six repeats in control alleles in all individuals under study (control and infected) at a time interval. In this study, the dominant allele was 4R, 5R, and 6R, among which 4R was the most frequent repeat (76.2% of ADHD patients and 53.8% of the control group) (P = 0.004) (21). Moreover, *DRD4* gene polymorphisms is a risk factor for children with attention deficit. Also, Bidwell observed that 4Rrepeats in the *DRD4* gene could be significantly related to ADHD (22). In the other study, Cheuk and Wong showed that the 4R allele was the most abundant repeat in their population with 48% abundance, and a significant relationship with ADHD was observed (7).

According to the studies above, although 4R repeat was a clear marker for ADHD, studies in other populations, especially European and American white populations, including the study of Nikolaidis and Gray, and the study of Leung et al., showed that 7R had a significant relationship with ADHD compared to other repeats (15, 19). In all of the above-mentioned studies that were investigated the associations, studies were performed in the form of case-control and intuitively, but the present study was a cohort study, and only the incidence of the above alleles was expressed descriptively in the present study. Therefore, we could not investigate the relationship in this study.

Treatment with methylphenidate had healing effects with increasing synaptic dopamine levels and tried to make up for the receptor's slow response. In the current study, it was observed that the presence of *DRD4* sub-types alleles in either homozygote or heterozygote forms could

not change the rate of reduction in patients' symptoms, and the intensity of symptoms did not relate with this subtype.

Jovanovic et al. suggested that there are no significant differences in presenting drugs or pharmacological cellular functions between the types of long and short repeats (23). Hamarman revealed that ADHD patients with seven repeats (7R) require higher doses of the drug for drug treatment (24). Also, McGough showed that there is a significant relationship between the gene and the dose of drug needed to control the disease (25).

Therefore, the *DRD4* promoter with P = 0.008 and *DRD4* exon III VNTR with P = 0.006 were associated with the intensity of symptoms after drug treatment approval. Surveying the above studies show that the relationship between gene and drug treatment in patients with ADHD is relative, and it is not observed in some studies. Subsequently, many causes are likely to be involved in the response of treatment in the patients, which is called meddler causes and leads to inconsistencies in the results of this study.

5.1. Conclusions

The results of this study show that the reduction of ADHD symptoms with drug treatment is not related to *DRD4* sub-types in patients with ADHD.

Footnotes

Authors' Contribution: All authors contributed to this research work. LMF wrote the paper and analyzed the data.

Conflict of Interests: All authors declared that there was no conflict of interest.

Ethical Approval: This research was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethics code IR.TBZMED.REC.1397.337).

Funding/Support: This research work was financed by Research Center of Psychiatry and Behavioral Sciences at Tabriz University of Medical Sciences.

Informed Consent: Written informed consent was provided by the patients.

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