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ASSOCIATION ANALYSIS OF FOUR HUMAN DOPAMINE PATHWAY GENES WITH ADULT ATTENTION-DEFICIT HYPERACTIVITY DISORDER IN A POPULATION FROM TURKEY

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In this tudy, it was aimed to investigate the association/s between dopamine transporter gene (DAT1)**,** dopamine receptor D1 (DRD1), dopamine receptor D2 (DRD2), dopamine receptor D3 (DRD3), dopamine receptor D4 (DRD4) gene variants and adult Attention Deficit and Hyperactivity Disorder (ADHD). A prospective analytical case control study. A total of 128 ADHD cases and 100 non-ADHD controls from Western population of Turkey were included in this study. DNA was isolated from peripheral blood. Genotype and allele frequency P-values were calculated by Chi square ($\chi 2$) and Fisher Exact tests. Other statistical analyses were carried out using SPSS program version 20.0. The genotypes for the DAT1, DRD2, DRD3 and DRD4 variants were identified by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). 4R allele and 4R/4R genotype of Exon 3 VNTR polymorphism in the DRD4 gene were observed to be the most frequent one in both case and control groups. 4R allele was found to be statistically significant in ADHD group than the ones in control group ($p=0.01$). No statistical differences in the genotype and allele frequencies were observed between ADHD cases versus non-ADHD controls for DAT1, DRD2 and DRD3 polymorphisms.

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A statistically significant association was found only between DRD4 Exon 3 VNTR polymorphism and adult ADHD. However, to confirm that these gene variants contributes to ADHD and ADHD-subtypes, further studies with both higher population sizes and many candidate genes are needed to be investigated simultaneously.

Key words: Adult Attention Deficit Hyperactivity Disorder (ADHD), Association analysis, Dopamine Pathway Genes, Polymorphism

INTRODUCTION

Attention Deficit and Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder with easy distraction of attention, difficulty in maintaining attention, impulsivity, hyperactivity, memory problems, bursts of anger, difficulty in controlling emotions. ADHD is one of the psychiatric diseases with the highest genetic transition (GOLDMAN *et al*., 1998). ADHD can be divided into three subtypes: ADHD predominantly inattentive subtype, ADHD predominantly hyperactive/impulsive subtype and ADHD combined subtype. Attention deficit subtype of ADHD, which is generally observed with carelessness, depression and anxiety disorders, is more common in girls. Symptoms of hyperactivity are prominent in boys and are often accompanied by devastating behavioral disorders such as aggression (ANGOLD *et al*., 2000; POLANCZYK *et al*., 2007).

It has been shown in longitudinal follow-up studies that ADHD symptoms continue in adulthood in 40-60% of people diagnosed with ADHD in childhood (MCGOUGH, 2005). Although ADHD symptoms vary from person to person in adults, symptoms related to attention deficit generally come to the fore. Due to the inability to concentrate on a job for a long time and misuse of time, it is seen as difficulty in completing the given job or delaying the works, forgetfulness, thoughtfulness, losing their belongings. Because of trying to execute more than one business at the same time and the flight of ideas, the planned works are generally inconclusive. These problems mostly affect academic life negatively and interpersonal communication (ERCAN, 2008; HECHTMAN, 2009; SENOL *et al*., 2008). The prevalence of lifelong ADHD decreases with age and the male/female ratio was found to be 1/1 in adulthood (ÖNER and ARSEV, 2007; WOLF and WASSERSTEIN, 2001). It is believed that the frequency of adult ADHD may be higher than the observed one due to the high frequency of comorbidity associated with ADHD and the fact that the studies could not be adapted to the general population due to studies conducted with selected samples (MARTIN, 2005).

Studies conducted to date about ADHD show that the most important factor in ADHD etiology is genetics. However, this genetic transition does not occur with a classical genetic transfer, but with a multifactorial inheritance. Many candidate gene studies have been conducted based on the knowledge of dysfunction in the catecholaminergic system in ADHD and the mechanism of action of psychostimulants used in the treatment of the disease. Molecular genetic studies have focused on the genes encoding the enzymes, receptors and polymorphisms in the catecholaminergic pathway (SWANSON *et al*., 2007). The main goal of methylphenidate used in the treatment of ADHD is to prevent the reuptake of dopamine from the synaptic area to the presynaptic terminal and ultimately increase the availability of extracellular dopamine (CASTELLANOS, 1997; VOLKOW and SWANSON, 2003). Genes involved in the activation of

dopamine and encoding dopamine receptors are located in the postsynaptic and presynaptic terminals. Five different dopamine receptor genes have been identified in the human genome. Dopamine receptor D1 (DRD1), dopamine receptor D2 (DRD2), dopamine receptor D3 (DRD3), dopamine receptor D4 (DRD4) and dopamine receptor D5 (DRD5) (FARAONE and BIEDERMAN, 1998). One of the genes thought to be responsible for the etiology of ADHD is the SLC6A3 (Solute Carrier Family 6 member 3) dopamine carrier gene, which encodes the dopamine transporter protein gene (DAT1). DAT 1 is located in the presynaptic neuron and takes part in the transport of dopamine from the cytoplasm to the vesicle (CURRAN *et al*., 2001). Stimulated drugs such as methylphenidate, which are known to have the most efficacy in the treatment of ADHD, show their effects on increasing the level of available dopamine in the synaptic space by inhibiting DAT1. Therefore, one of the most studied genes related to the dopaminergic system is the DAT1 gene (VOLKOW *et al*., 1995; VOLZ, 2008). Polymorphisms selected as candidate genes because it is assumed to predispose adult ADHD development were as follows: DAT1 gene 3'UTR VNTR polymorphism, DRD2 gene *Taq*I A polymorphism (rs1800497), DRD3 gene Ser9Gly polymorphism (rs6280), DRD4 exon 3 VNTR polymorphism (rs6280), DRD4 exon 3 VNTR polymorphism.

The aim of our study was to evaluate the relationship between the DAT1, DRD2, DRD3 and DRD4 gene polymorphisms and ADHD and ADHD-subtypes in an adult population from Western Turkey as a case-control survey.

MATERIALS AND METHODS

Our study was conducted between May 2016 to August 2017, including 128 patients who were admitted to Afyon Kocatepe University (currently Afyonkarahisar Health Sciences University) Faculty of Medicine Psychiatry Outpatient Clinic and diagnosed with ADHD, and 100 healthy individuals. Our study was designed as a case-control study.

Subjects

A total of 128 adult ADHD cases (70 men and 58 women) between the ages 18–65 years from Afyon Kocatepe University Hospital (currently Afyonkarahisar Health Sciences University Hospital) were included in the present study. In addition, 100 healthy age matched controls (55 men and 45 women) were recruited. All subjects were interviewed by a Psychiatrist. Consensus diagnoses were made according to DSM- IV (The Diagnostic and Statistical Manual of Mental Disorders) Diagnostic Criteria for ADHD. Our study was explained to the people who met the inclusion criteria after psychiatric pre-interview and written consent was obtained from all subjects. Subjects with neurological disease, psychotic disorder, organic psychiatric disorders and mental retardation accompanying ADHD were excluded from the study.

From the total of 128 cases included in the study, 15 of which were ADHD-Attention Deficit Dominant Type, 38 were ADHD-Hyperactivity-Impulsiveness Dominant Type and 75 ADHD-Combined (Combined) Type.

Polymerase Chain Reaction and Genotyping

For genomic DNA isolation, 200 µl peripheral blood samples were prepared and genomic DNA's were extracted from it using QuickGene-Mini80 (Kurabo, JAPAN) genomic

DNA isolation kit according to Manufacturer's instruction and then used as a template for PCR.

DAT1 3′ VNTR polymorphism

The PCR primers for DAT1 3′ VNTR polymorphism were as follows: Forward 5′- GGT CCT TGT GGT GTA GGG AA -3′ and Reverse 5′- CGC AAA CAT AAA AAC TGT TGT T -3′ (HUANG *et al*., 2010). The PCR amplification was performed on the thermal cycler as follows: an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles consisting of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes. Analysis of the amplified products were then done on agarose gel electrophoresis (%2) stained with ethidium bromide in 0.5x Tris-EDTA (ethylenediamine tetraaceticacid)-borate buffer (TBE) along with a 100 bp DNA ladder (Axygen) followed by visualization under UV light. The genotypes for DAT1 3′ VNTR polymorphism were determined depending on the product size obtained along with a 100 bp DNA marker (Axygen) as follows: 598-bp: 11R, 558-bp:10R, 515-bp:9R, 478-bp:8R as shown in Fig 1. Afterwards genotype distributions and allele frequencies were calculated for both ADHD cases and controls.

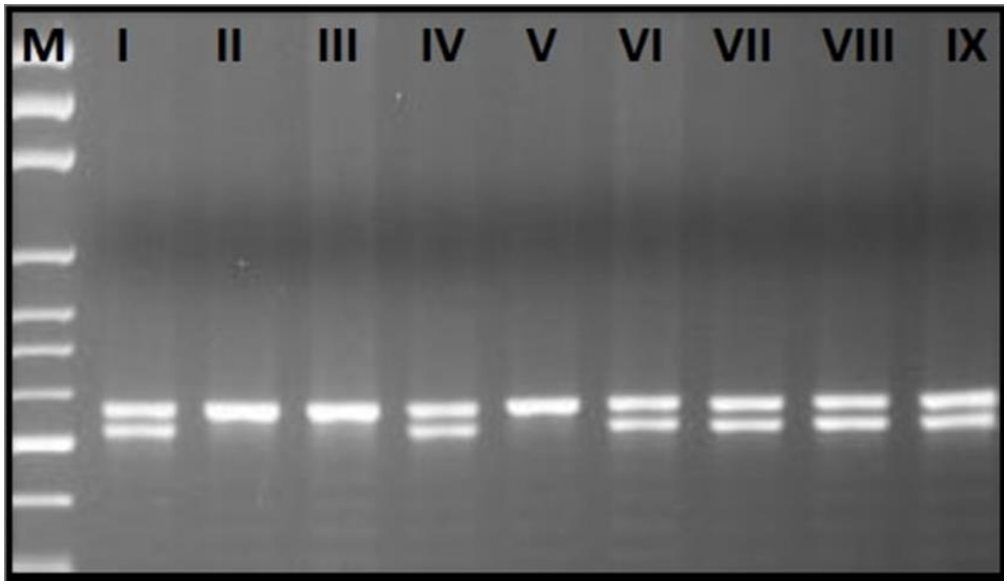

Fig 1. PCR detection of the DAT1 gene 3'UTR 40 bp VNTR polymorphism by an agarose gel electrophoresis (2%) followed by ethidium bromide staining and UV transullumination was performed to visualize and genotype the PCR products together with 100 base pair (bp) DNA marker (Axygen Biosciences). The most commonly observed product sizes were: homozygotes (10R/10R or 9R/9R) having either 558 or 518 bp bands respectively; heterozygotes (10R/9R) having both 558 bp or 518 bp bands. 10R/10R: 10 Repeat/10 Repeat genotype (lanes 2, 3, 5), 9R/9R: 9 Repeat/9 Repeat genotype (not shown here), 10R/9R: 10 Repeat/9 Repeat genotype (lanes 1, 4, 6, 7, 8, 9), D: Deletion allele, I: Insertion allele, M: Marker or DNA Ladder (lanes 1).

DRD2 *Taq*I A polymorphism

The PCR primers for DRD2 *Taq*I A polymorphism were as follows: Forward 5′-AGG TGT CTT GGA TTG GAG A -3′ and Reverse 5′- CCT TCC TGA GTG TCA TCA -3′ (GRANDY *et al*., 1993). The PCR amplification was performed on the thermal cycler as follows: an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles consisting of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes. The PCR products amplified then were digested using *TaqI* restriction enzyme at 65°C for three hours to genotype DRD2 *Taq*I A polymorphism variants. Analysis of the amplified products were then done on agarose gel electrophoresis (%2) stained with ethidium bromide in 0.5x Tris-EDTA (TBE) along with a 100 bp DNA ladder (Oxygen) followed by visualization under UV light. The genotypes for DRD2 *Taq*I A polymorphism were determined depending on the product size obtained along with a 100 bp DNA marker (Oxygen) as follows: The ones having 780-bp and 126-bp bands were genotyped as CC, the ones that have only one band of 906-bp were genotyped as TT and the ones having both alleles were genotyped as CT as can be seen in Fig 2. Afterwards genotype ditributions and allele frequencies were calculated for both ADHD cases and controls.

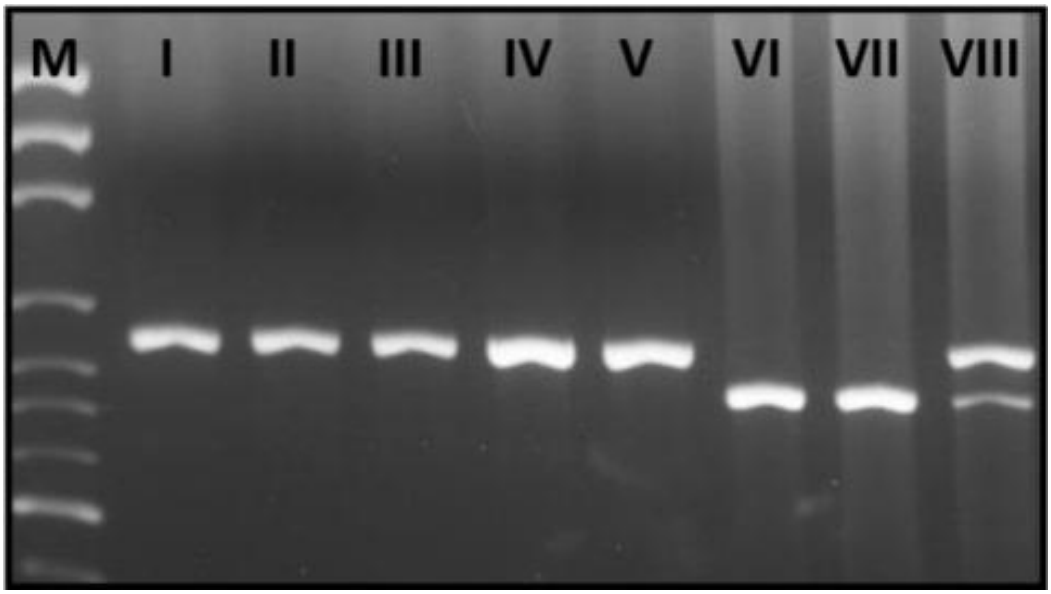

Fig 2. Image of the PCR product containing the DRD2 gene *TaqI* A polymorphism (rs1800497) on a 2% agarose gel after cutting with *TaqI* enzyme followed by ethidium bromide staining and UV transullumination was performed to visualize and genotype the PCR products together with 100 base pair (bp) DNA marker (Axygen Biosciences). The observed product sizes were: homozygotes (C/C or T/T) having either 780 or 906 bp bands respectively; heterozygotes (C/T) having both 780 bp and 906 bp bands. CC genotype (lanes 6 and 7), TT genotype (lanes 1-5), CT genotype (lane 8). C: Cytosine allele, T: Thymine allele, M: Marker or DNA Ladder.

DRD3 Ser9Gly polymorphism

The PCR primers for DRD3 Ser9Gly polymorphism were as follows: Forward 5′- GTG TTG TTC TCA CTG CTC AG -3′ and Reverse 5′- AAG TCT ACT CAC CTC CAG GTA -3′ (BALLON *et al*., 2007). The PCR amplification was performed on the thermal cycler as follows: an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles consisting of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes. The PCR products amplified then were digested using *HaeIII* restriction enzyme at 37°C for one hour to genotype DRD3 Ser9Gly polymorphism variants. Analysis of the amplified products were then done on agarose gel electrophoresis (%2) stained with ethidium bromide in 0.5x Tris-EDTA (TBE) along with a 100 bp DNA ladder (Oxygen) followed by visualization under UV light. The genotypes for DRD3 Ser9Gly polymorphism were determined depending on the product size obtained along with a 100 bp DNA marker (Oxygen) as follows: The ones having only 500-bp band was genotyped as GG and 550-bp band was genotyped as AA and the ones having both alleles were genotyped as AG as shown in Fig 3. Afterwards genotype distributions and allele frequencies were calculated for both ADHD cases and controls.

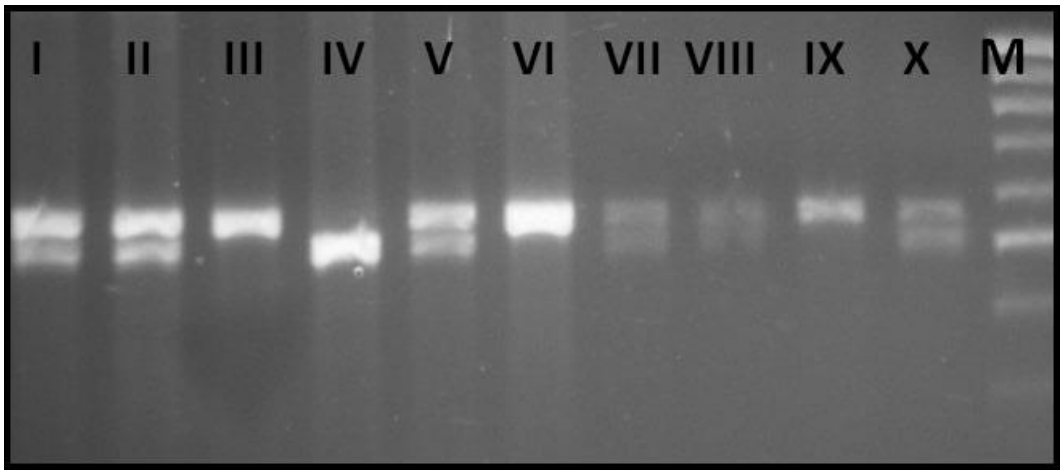

Fig 3. PCR amplification of the DRD3 gene Ser9Gly polymorphism (rs6280) by an agarose gel electrophoresis (2%) after cutting with *HaeIII* enzyme followed by ethidium bromide staining and UV transullumination was performed to visualize and genotype the PCR products together with 100 base pair (bp) DNA marker (Axygen Biosciences). The observed product sizes were: homozygotes (A/A or G/G) having either 550 or 500 bp bands respectively; heterozygotes (A/G) having both 550 bp and 500 bp bands. AA: Adenine (Serine)/Adenine (Serine) genotype (lanes 3, 6, 9), GG: Guanine (Glycine)/Guanine (Glycine) genotype (lanes 4), AG: Adenine (Serine)/Guanine (Glycine) genotype (lanes 1, 2, 5, 7, 8, 10), A: Adenine (Serine) allele, G: Guanine (Glycine) allele, M: Marker or DNA Ladder (last lane).

DRD4 Exon 3 VNTR polymorphism

The PCR primers for DRD4 Exon 3 VNTR polymorphism were as follows: Forward 5′ - GCG ACT ACG TGG TCT ACT CG -3′ and Reverse 5′ - AGG ACC CTC ATG GCC TTG -3′ were used as well as other components (LICHTER *et al.*, 1993). The PCR amplification was performed on the thermal cycler as follows: An initial denaturation occurred at 96°C for 5 minutes, and the three-phase extension process at 96°C for 45 seconds, 59°C for 45 seconds and 72°C for 45 minute was carried out for 36 cycles. Then, the final extension was performed at 72°C for 10 minutes. Analysis of the amplified products were then done on agarose gel electrophoresis (%2) stained with ethidium bromide in 0.5x Tris-EDTA (TBE) along with a 100 bp DNA ladder (Oxygen) followed by visualization under UV light. The genotypes for DRD4 Exon 3 VNTR polymorphism were determined depending on the product size obtained along with a 100 bp DNA marker (Oxygen) as follows: 378-bp:2R, 426-bp:3R, 474-bp:4R, 522-bp:5R, 570-bp:6R as shown in Fig 4. Afterwards genotype distributions and allele frequencies were calculated for both ADHD cases and controls.

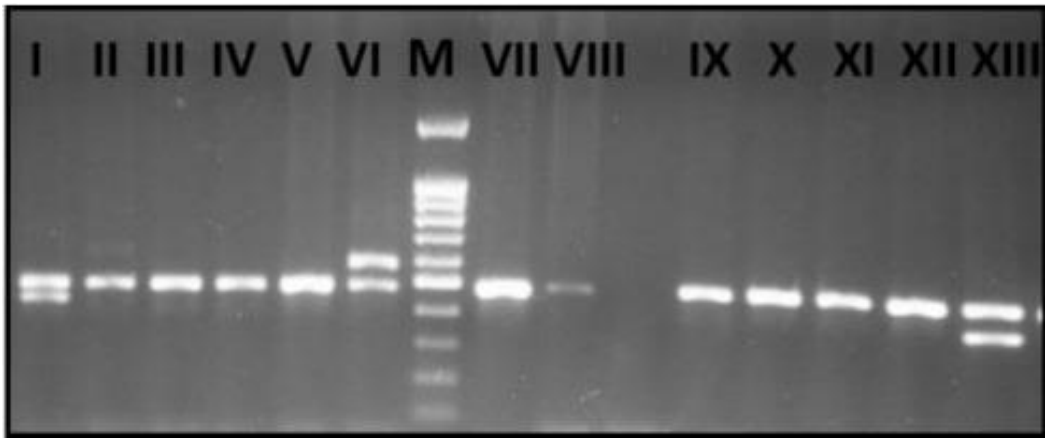

Fig 4. PCR detection of the DRD4 gene exon 3 48 bp VNTR polymorphism by an agarose gel electrophoresis (2%) followed by ethidium bromide staining and UV transullumination was performed to visualize and genotype the PCR products together with 100 base pair (bp) DNA marker (Axygen Biosciences). The most commonly observed product sizes were: homozygotes (4R/4R genotype) having a 474 bp band seen in lanes 2, 3, 4, 5, 7, 8, 9, 10, 11, 12; heterozygotes (4R/3R genotype) having both 474 bp and 426 bp bands (lane 1); (4R/6R genotype) having both 474 bp and 570 bp bands (lane 6) and as well as (4R/2R genotype) having both 474 bp and 378 bp bands (lanes 13). 4R: 4 Repeat allele, 3R: 3 Repeat allele, 6R: 6 Repeat allele, 2R: 2 Repeat allele, M: Marker or DNA Ladder.

Statistical Analysis

Data were analyzed using Statistical Program for Social Science (SPSS) version 20.0[22]. Chi square ($\chi 2$) test of significance was used in order to compare proportions between 2 qualitative parameters such as genotype distributions among case group versus controls and $\chi 2$ test for allele frequencies to see if there is any significance between cases and controls. Probability (P value) < 0.05 was considered significant.

Ethics

This study was submitted to Dumlupınar University Clinical Research Ethics Committee for approval and it was obtained with the decision number 2015-KAEK-86 / 05-87.

RESULTS

To test that if there is an association between the four different gene polymorphisms (DAT1 gene 3′ VNTR, DRD2 gene *Taq*I A, DRD3 gene Ser9Gly and DRD4 gene Exon 3 VNTR polymorphisms) and adult ADHD, a total of 128 ADHD cases and 100 control subjects were genotyped and the genotype distributions and allele frequencies were calculated. Then analysis of association was performed whether there is any differences between cases and controls in terms of genotype distributions and allele frequencies.

For the DAT1 3′ VNTR polymorphism, genotype distribution was 10R/10R = 60%, 9R/10R = 41%, 9R/9R = 0.1 % in cases and 10R/10R = 43%, 9R/10R = 43%, 9R/9R = 0.8% in controls (Table 1) Genotype frequencies for this polymorphism accorded with Hardy–Weinberg equilibrium. There was a marginal statistical difference in respect to genotype distribution (P= 0.058) and no difference between allele frequencies (p= 0.09) of the DAT1 gene 3′ VNTR polymorphism among ADHD cases versus controls. Besides, 10R/10R genotype was seen the most frequently in both cases and controls, while the 9R/9R genotype was seen the least frequently in both groups. The 10R allele was significantly more common in the ADHD group than the control group (76%, 69%). More observation of 10R/10R genotype and 10R allele in the case group suggests that this allele may be a risk allele for ADHD. Five alleles of the DAT1 gene 3′ VNTR (7R, 8R, 9R, 10R and 11R) were detected. 9R and 10R were the most predominant genotypes both among ADHD cases and control subjects. For the DAT1 3′ VNTR, the 10R allele was the most common (frequent) (73%), followed by the 9R (23%) among the ADHD cases and (68%), followed by the 9R (30%) among the controls. The genetic distribution of 3′ VNTR polymorphism of DAT1 coincided with the expected values of the Hardy-Weinberg Equilibrium. Of the other three rare alleles, the 7R allele was observed in 3 out of 128 ADHD samples, 2 out of 100 control subject showed this allele. The other rare allele (8R) were observed only in 2 out of 128 ADHD samples versus only one in control subjects of 100. Also, 11R allele was observed 3 out of 128 ADHD samples, only one out of 100 control showed this allele. The frequencies of all the rare alleles were lower than 1% (Table 1). No significant differences in allele frequency distributions were detected between ADHD cases and non-ADHD controls in regard to 7R, 8R and 11R alleles, though 10R allele frequency in cases and 9R allele frequency were higher in controls respectively.

Table 1. Genotype distributions and allele frequencies of DAT1 3'UTR 40-bp VNTR polymorphism in ADHD and control group

	ADHD	Control	
Genotype Frequencies	N (%)	N (%)	P value**
9R/9R	2 (0.01)*	8 (0.083)	0.058
9R/10R	52 (0.41)	43 (0.45)	NS
10R/10R	65 (0.60)	45 (0.47)	NS
Total	119 (100.0%)	96 (100.0%)	NS
Allele Frequencies	N (%)	N (%)	P value***
Allele 1 7R	3/254 (0.01)*	2/200 (0.031)	NS
Allele 2 8R	2/254 (0.00)	1/200 (0.005)	NS
Allele 3 9R	60/254 (0.23)	60/1200 (0.30)	NS
Allele 4 10R	186/254 (0.73)	136/200 (0.68)	0.22 (NS)
Allele 5 11R	3/254 (001)	1/200 (0.000)	NS
Total	254 (100.0%)	200 (100.0%)	NS

*Genotype and allele data were given as numbers and percentages (genotype and allele frequency). **Genotypic *P*-values were calculated by χ^2 (2 df) and allele frequency *P*-values were calculated by Fisher's exact test, 1-tailed test and *P*-values of <0.05 between compared groups were regarded as significant statistically. (NS: Not Signifiant; ADHD: Attention Deficit Hyperactivity Disorder)

To test that if there is an association between the DRD2 gene *Taq*I A polymorphism and ADHD, a total of 128 ADHD cases and 100 control subjects were genotyped and the genotype distributions and allele frequencies were calculated. Then analysis of association was performed whether there is any differences between cases and controls in terms of genotype distributions and allele frequencies. Genotype distribution of *Taq*I A polymorphism was C/C=71%, C/T=25%, T/T=4% in cases and C/C=73%, C/T=25%, T/T=0.02% in controls. Genotype frequencies for this polymorphism accorded with Hardy–Weinberg equilibrium. There was no statistical difference in respect to genotype distribution (p=0.60) and allele frequencies (p=0.70) of the DRD2 gene *Taq*I A polymorphism among ADHD cases versus controls. Besides, CC genotype was seen the most frequently in both cases and controls, while the TT genotype was seen the least frequently in both groups. The allele frequencies in both groups were very close to each other and there was no significant difference among the two groups (Table 2).

To test the DRD3 gene Ser9Gly polymorphism for association with ADHD, a total of 128 ADHD cases and 100 control subjects were genotyped and the genotype distributions and allele frequencies were calculated. Then analysis of association was performed whether there is any difference between cases and controls in terms of genotype distributions and allele frequencies. Genotype distribution of Ser9Gly polymorphism was A/A(S/S)=48%, A/G(S/G)= 42%, G/G(G/G)= 10% in cases and A/A= 58%, A/G=34%, G/G= 0.08% in controls. Genotype frequencies for this polymorphism accorded with Hardy–Weinberg equilibrium. There was no statistical difference in respect to genotype distribution (p= 0.299) and allele frequencies (p= 0.142) of the DRD3 gene Ser9Gly polymorphism among ADHD cases versus controls. Besides, A/A genotype was seen the most frequently in both cases and controls, while the G/G genotype

was seen the least frequently in both groups. The allele frequencies in both groups were very close to each other and there was no significant difference among the two groups (Table 3).

Table 2. Genotype distributions and allele frequencies of DRD2 gene TaqI A polymorphism in ADHD and control group.

	n	Genotype* CC	CT	TT	χ^2	P-value**	Allele* C	T	P-value**	HWL***
ADHD	128	91 (0.71)	32 (0.25)	5 (0.4)			214/256 (0.836)	42/256 (0.164)		0.84
Control	100	73 (0.73)	25 (0.25)	2 (0.02)	0.693	0.70	171/200 (0.855)	29/200 (0.145)	0.60	0.86

*Genotype and allele data are given as numbers and percentages (genotype and allele frequency).

**Genotype *P*-values were calculated by χ^2 (2 df) and allele frequency *P*-values were calculated by Fisher's exact test, 1-tailed test and *P*-values of <0.05 between compared groups were regarded as significant statistically. ***HWE: Hardy-Weinberg Law (Equilbirium). (ADHD: Attention Deficit Hyperactivity Disorder)

Table 3. Genotype distributions and allele frequencies of DRD3 gene Ser9Gly polymorphism (rs6280) in ADHD and control group

	n	Genotype* AA (S/S)	AG (S/G)	GG (G/G)	χ^2	P-value**	Allele* A (Ser)	G (Gly)	P-value**	HWL***
ADHD	128	61 (0.48)	54 (0.42)	13 (0.10)			176/256 (0.687)	80/256 (0.312)		0.69
					2.409	0.299			0.142	
Control	100	58 (0.58)	34 (0.34)	8 (0.08)			150/200 (0.75)	50/200 (0.25)		0.75

*Genotype and allele data are given as numbers and percentages (genotype and allele frequency).

**Genotype *P*-values were calculated by χ^2 (2 df) and allele frequency *P*-values were calculated by Fisher's exact test, 1-tailed test and *P*-values of <0.05 between compared groups were regarded as significant statistically. ***HWE: Hardy-Weinberg Law (Equilbirium). (ADHD: Attention Deficit Hyperactivity Disorder)

To test that if there is an association between the DRD4 gene Exon 3 VNTR polymorphism and ADHD, a total of 81 ADHD cases and 63 control subjects were genotyped and the genotype distributions and allele frequencies were calculated. Then analysis of association was performed whether there are any differences between cases and controls in terms of genotype distributions and allele frequencies. Distribution of DRD4 Exon 3 VNTR polymorphism was 4R/4R= 84%, 2R/4R= 0.08%, 2R/2R= 0.08% in cases and 4R/4R= 79%, 2R/4R= 112%, 2R/2R= 0.08% in controls. Genotype frequencies for this polymorphism accorded with Hardy–Weinberg equilibrium. There was no statistical difference in respect to genotype distribution (P= 0.71) of the DRD4 gene Exon 3 VNTR polymorphism among ADHD cases versus controls (Table 4). Besides, 4R/4R genotype was seen the most frequently in both cases and controls, while the

2R/2R genotype was seen the least frequently in both groups. In both groups, the 4R allele was the most common allele than other repeat alleles, and was statistically significant being higher in the ADHD group than the controls (p= 0.01). More observation of 4R/4R genotype and 4R allele in the case group suggests that this allele may be a risk allele for ADHD. Seven alleles of the DRD4 gene Exon 3 VNTR polymorphism (1R, 2R, 3R, 4R, 5R, 6R and 7R) were detected. 2R and 4R alleles were the most predominant ones both among ADHD cases and control subjects. For the DRD4 Exon 3 VNTR polymorphism, the 4R allele was the most common (frequent) (86%), followed by the 2R (9%) among the ADHD cases and (74%), followed by the 2R (7%) among the controls. The genotype distribution of Exon 3 VNTR polymorphism of DRD4 coincided with the expected values of the Hardy-Weinberg Equilibrium. No significant differences in allele frequency distributions were detected between ADHD cases and non-ADHD controls in regard to rare 1R, 3R, 5R, 6R and 7R alleles (Table 4).

Table 4. Genotype distributions and allele frequencies of DRD4 exon 3 48-bp VNTR polymorphism in ADHD and control group

	ADHD	Control	
Genotype Frequencies	N (%)	N (%)	P value*
2R/2R	6 (0.08)*	4 (0.08)	0.71
2R/4R	6 (0.08)	6 (0.12)	
4R/4R	65 (0.084)	39 (0.79)	
Total	77 (100.0%)	49 (100.0%)	
Allele Frequencies**	N (%)	N (%)	P value**
Allele 1 (330 bp) 1R	0/162 (0.00)	4/126 (0.031)	NS
Allele 2 (378 bp) 2R	18/162 (0.90)	17/126 (0.134)	NS
Allele 3 (426 bp) 3R	2/162 (0.123)	5/126 (0.039)	NS
Allele 4 (474 bp) 4R	140/162 (0.864)	94/126 (0.746)	0.01
Allele 5 (522 bp) 5R	1/162 (0.006)	0/126 (0.000)	NS
Allele 6 (570 bp) 6R	1/162 (0.006)	5/126 (0.039)	NS
Allele 7 (618 bp) 7R	0/162 (0.000)	1/126 (0.008)	NS
Total	162 (100.0%)	126 (100.0%)	NS

*Genotype and allele data were given as numbers and percentages (genotype and allele frequency). **Genotypic *P*-values were calculated by χ^2 (2 df) and allele frequency *P*-values were calculated by Fisher's exact test, 1-tailed test and *P*-values of <0.05 between compared groups were regarded as significant statistically. (NS: Not Signifiant; ADHD: Attention Deficit Hyperactivity Disorder)

DISCUSSION

In our study, it was investigated whether there was a relationship between ADHD and four different gene variants. A study designed as a case-control study included 128 cases diagnosed with ADHD as a result of psychiatric interview and 100 control individuals not diagnosed with ADHD. There was no significant difference between the case group and the control group in terms of average age, which is one of the sociodemographic features. When we evaluated in terms of gender, 45.3% of the ADHD group consisted of women and 54.6% consisted of men. The gender distribution of the case group included in our study was closer to the ratio of women and men reported in adult ADHD. There was no significant difference between the ADHD group and the control group included in our study in terms of the years of education they received. Although there is a study showing that the educational level of ADHD cases is lower than healthy controls, such a difference was not observed in both our own and other studies (Kessler et al., 2006; Mcgough, 2005). Three subtypes of ADHD were defined in DSM-IV: ADHD-predominantly inattentive subtype, ADHD-predominantly hyperactive/impulsive subtype and ADHD-combined type. In our study, 15 (11.7%) of the individuals diagnosed with ADHD-predominantly inattentive subtype, 38 (29.6%) ADHD-predominantly hyperactive/impulsive subtype and 75 (58.5%) ADHD-combined type were observed. The most common type in our study was the combined type in both genders. While inattentive subtype was more common in women, hyperactive/impulsive subtype was more common in men. The distribution of individuals among the types observed in our study was compatible with the one reported in the literatüre (CURRAN and TAYLOR, 2000; GRAETZ *et al*., 2005; RAMUSSEN *et al*., 2002; SENOL *et al*., 1994).

In our study, genotype distributions and allele frequencies of four different polymorphisms in DAT1, DRD2, DRD3 and DRD4 genes in ADHD and non-ADHD control individuals were compared. Although the studies on similar gene polymorphisms in the literature and ADHD produced contraversial results; in our study, some findings are compatible with the literature and some are found to be slightly different. This may be due to the heterogeneity in genetic and environmental factors in Afyonkarahisar province in Turkey. However, one of the most important limitations of our study is that the number of cases and control groups are 128 and 100 respectively. The ADHD cases included in the study were selected among those who applied to the psychiatry clinic. The reason for being the number of cases were low might be due to the fact that the adult ADHD diagnosis frequency was much lower than the incidence in the population. Therefore, these findings should be confirmed further by other studies containing more samples. In addition, studies on all other candidate genes will also be useful in illuminating the genetic factors that play a role in the studied population. Nonetheless, our study could be an introduction to adult ADHD genetics in Turkey.

DAT1 gene 3' VNTR polymorphism

The first study that investigated the relationship between ADHD and DAT1 gene and found that some polymorphisms in the DAT1 gene predispose to ADHD was conducted by COOK *et al*. (1995). Although there are contradictory results, the candidate gene studies conducted prove the DAT1-ADHD relationship. Although numerous candidate gene study conducted in children with ADHD, studies on the genetics of adult ADHD is limited both in Turkey and the world.

According to the responses to methylphenidate treatment, 42 adult ADHD of Caucasian origin, there was no difference in DAT1 genotypes (KOOJI *et al*., 2008). In a study conducted in 171 adult ADHD cases of Brazilian origin, the most common allele was stated 10R allele, but there was no significant difference between the alleles in terms of the methylphenidate response (CONTINI et al., 2010). Azeredo et al. showed that 10R/10R genotype was the most common genotype in both groups in the study comparing 522 adult ADHD patients and 628 healthy adults (AZEREDO *et al*., 2014). However, they reported that DAT1 expression decreased in those with the 9R/10R genotype, which could have a protective effect against ADHD by increasing dopamine in the synaptic area. In the study on 77 adult ADHD and 474 healthy controls of European origin, they stated that there was a significant difference in the control and case group in terms of allele frequency and the newly described 9.5R allele was less in the control group and had a protective effect (HASLER *et al*., 2015).

In accordance with the literature in our study, the most common genotype in both groups is 10R/10R, while the relatively less common genotype is 9R/9R. It is seen that there is a statistically marginal difference in terms of genotype distributions and the 10R/10R genotype is higher in the ADHD group (P= 0.058). Although the 10T allele was not statistically significant in terms of allele frequencies, it was observed more in the ADHD group than in the case group. The only study investigating the association between adult ADHD and DAT1 gene was carried out with 79 patients and 75 control in Turkey and there is no relationship between the adults with ADHD and DAT1 according to the study (Sevinc et al., 2010). More sample studies on adult ADHD geneticsin Turkey are needed.

DRD2 gene TaqI A polymorphism

Compared to other dopamine receptors, genetic studies investigating the relationship between DRD2 and ADHD are limited. It is stated that this gene plays a role as a modifying factor in ADHD rather than an etiological factor. It is not certain whether this gene plays a role in the etiology of ADHD, as the studies conducted so far investigating the association between DRD2 and ADHD yield different results (ROWE *et al.*, 1999). A family-based study on 93 ADHD children in Taiwan found no significant relationship between *Taq*I A polymorphism in the DRD2 gene and ADHD (HUANG *et al*., 2003). In another study conducted on 535 children with Caucasian origin, no relationship was found between DRD2 *Taq*I A polymorphism and ADHD (KUSTANOVICH *et al*., 2004).

In addition, a study conducted with 178 ADHD and 157 control youth, only a significant relationship was observed between the mentioned DRD2 variant and ADHD in women and not in men (NYMAN *et al*., 2012). Sery et al. found a statistically significant difference between the DRD2 variant and ADHD in their study with 70 boys with ADHD- hyperactive/impulsive subtype and 108 controls in the Czech Republic. It was shown that the frequency of A1 allele was significantly higher in the case group, and individuals with the A1/A1 genotype were more frequent in the case group than the controls (SERY *et al*., 2006).

The results of our study are compatible with studies that found no relationship between the DRD2 *Taq*I A polymorphism and ADHD on children. Our findings do not support the role of the DRD2 gene in the development of adult ADHD in the population studied. Our study is the first

study in Turkey investigating the relationship between the DRD2 gene and ADHD in the adult population.

DRD3 gene Ser9Gly polymorphism

Studies investigating the relationship between DRD3 and ADHD in the adult population are few and the results are contradictory. In a study on 39 adult ADHD, no significant difference was found between DRD3 gene Ser9Gly polymorphism and ADHD (MUGLIA *et al*., 2002). In the study on 146 adult men, the relationship between impulsivity and DRD3 was investigated and a significant relationship was found between DRD3 Ser9Gly polymorphism. Men with heterozygous A1/A2 genotypes have been shown to be more impulsive than those with homozygous genotypes (A1/A1 or A2/A2) (RETZ *et al*., 2003). Investigating the association between DRD3 gene and adults ADHD, there is only one study in Turkey, no significant relationship was found similar to our study (SEVINC *et al*., 2020). At least we can say that this polymorphism is not effective in the development of ADHD in the population we study or in the overall Turkish population considering both reports. However, since the number of adult samples includedwas low, more studies with larger samples may need to be repeated.

DRD4 gene Exon 3 VNTR polymorphism

In the studies on ADHD, the frequency of allele in the 48-bp VNTR polymorphism of the DRD4 gene in exon 3 varies in different ethnic groups. In various studies conducted in different countries, although the frequency of the allele varies in ADHD cases, the most common alleles have been shown to be the 4R allele and the 7R allele, respectively (LI *et al*., 2006; SEVINC *et al*., 2010; SHAW *et al*., 2007) In a family-based study on 50 Turkish children with ADHD, a significant correlation was found between 7R polymorphism and ADHD, and this relationship was found to be stronger in 24 children with ADHD who responded positively to psychostimulant therapy (TAHIR *et al*., 2000). In some case-control studies investigating the relationship between the DRD4 4R allele and ADHD, the 4R allele has been shown to be lower in ADHD compared to the controls, so the 4R allele was thought to have a protective effect for ADHD (LI *et al*., 2006; SHAW *et al*., 2007). Another study reported that individuals with 4R/4R homozygous genotypes respond better to treatment (MCGOUGH *et al*., 2006). In a study on 50 children with ADHD in Egypt, the 4R allele was found to be higher statistically significant in the case group and it was reported that it may be a risk allele (ELBAZ-MOHAMED *et al*., 2017). In a study conducted with 130 children with ADHD in Iran, the 4R allele was the most common allele in both groups and was higher significantly in the case (TABATABAEI *et al*., 2017).

There are different outcomes in ADHD studies in Turkey. There was no significant difference between the groups in terms of 4R allele frequencies compared with 200 ADHD cases and 100 healthy children. When the frequency of 4R/4R genotype was compared, the frequency of 4R/4R genotype was found higher in ADHD-Combined type than control and other subtypes (BACANLI, 2011). In the case-control study conducted with 79 adult ADHD by Sevinç et al., they showed that the most common allele in the DRD4 gene in both groups was the 4R allele, but there was no statistically significant difference between the two groups. They also found that individuals with the 4R/4R genotype respond better to treatment. However, the 4R allele frequency was higher significantly in the case group (P= 0.01). Similar to previous studies, the

4R allele and the 4R/4R genotype in our study are the most common allele and genotype respectively in both groups.

This suggests that, unlike other results, this allele may be a risk allele for ADHD. It is clear that further studies with larger samples are needed to reveal the relationship between DRD4 gene Exon 3 VNTR polymorphism and ADHD more clearly. Considering the studies carried out to date, it is still not clear which gene is in which state and in what rate and how interacts with ADHD. In fact, another factor to be considered is how environmental factors affect genetic risks. Studies focused mostly on patients with genetic risk. Although a family carries disease susceptibility genes, the properties associated with disease genes found in non-sick members have not been investigated. Oligogenic genetic model in ADHD, ethnic differences, environmental factors, presence of subtypes and comorbidities; genetic differences are the causes of different results in genetic studies. As a result, a genetic polymorphism alone is not sufficient in the development of this disease, and therefore a positive relationship may not always be detected since many genes might be contributing. If there is a relationship between adult ADHD and a gene polymorphism, future genetic studies are needed to reveal how and to what extent this polymorphism has an impact on the ADHD development process. Moreover, it needs to be confirmed by further studies with larger sample size to reveal its exact role in ADHD both in the country detected and other countries before the results are accepted as conclusive.

CONCLUSION

In conclusion, we studied four gene polymorphisms (three in dopamine receptor genes which were DRD2, DRD3, DRD4 and one in a dopamine transporter gene, DAT1). As a result, we found that the Exon 3 VNTR polymorphism in the DRD4 gene was associated with ADHD. Therefore, this study presents evidence which supports the role of DRD4 gene Exon 3 VNTR polymorphism in ADHD. Besides, this is the first report showing an association of DRD4 Exon 3 VNTR polymorphism with ADHD in an adult population from Turkey. However, it needs to be confirmed further in larger populations to reveal its exact role in ADHD.

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ASOCIJATIVNA ANALIZA ČETIRI HUMANA GENA DOPAMINSKOG PUTA SA HIPERAKTIVNIM POREMEĆAJEM PAŽNJE ODRASLIH U POPULACIJI IZ TURSKE

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Izvod

U ovoj studiji, cilj je bio da se ispita povezanost/e veze između varijanti gena dopaminskog transportera (DAT1), dopaminskog receptora D1 (DRD1), dopaminskog receptora D2 (DRD2), dopaminskog receptora D3 (DRD3), dopaminskog receptora D4 (DRD4). i poremećaja pažnje i hiperaktivnosti odraslih (ADHD). Prospektivna analitička studija kontrole slučajeva. U ovu studiju je uključeno ukupno 128 slučajeva ADHD-a i 100 kontrola bez ADHD-a iz zapadne populacije Turske. DNK je izolovana iz periferne krvi. P-vrednosti učestalosti genotipa i alela izračunate su Hi kvadratom ($\chi 2$) i Fisher Ekact testovima. Ostale statističke analize su sprovedene korišćenjem SPSS programa verzije 20.0. Genotipovi za varijante DAT1, DRD2, DRD3 i DRD4 su identifikovani polimeraznom lančanom reakcijom praćenom polimorfizmom dužine restrikcionog fragmenta (PCR-RFLP). Uočeno je da su 4R alel i 4R/4R genotip polimorfizma Ekon 3 VNTR u DRD4 genu najčešći u oba slučaja iu kontrolnoj grupi. Utvrđeno je da je alel 4R sitatistički značajan u ADHD grupi od onih u kontrolnoj grupi (p=0,01). Nisu primećene statističke razlike u učestalosti genotipa i alela između slučajeva ADHD u odnosu na kontrole bez ADHD za polimorfizme DAT1, DRD2 i DRD3. Statistički značajna povezanost pronađena je samo između polimorfizma DRD4 Ekon 3 VNTR i ADHD-a odraslih. Međutim, da bi se potvrdilo da ove varijante gena doprinose ADHD-u i ADHD-podtipovima, potrebno je istovremeno istraživati dalje studije sa većim veličinama populacije i mnogim genima kandidata.

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