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Association study between the dopamine-related candidate gene polymorphisms and ADHD among Saudi Arabia population via PCR technique

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Abstract Attention deficit hyperactivity disorder (ADHD) is one of the most common childhood behavioral disorders characterized by inattention, hyperactivity and impulsivity. In Saudi Arabia the prevalence of combined ADHD is 16.4 %. ADHD etiology is not clear and not completely understood. There are several evidences for involvement of dopaminergic, serotonergic and noradrenergic neurotransmitter systems in the pathogenesis of ADHD. Monoamine Oxidase A (MAOA) is involved in the degradation of all three of these neurotransmitters. Dopamine Transporter 1 (DAT1) plays an important role in controlling blood levels of dopamine. The aim of the present study is to investigate the association between ADHD and polymorphisms of MAOA 30 bp-promoter VNTR and DAT1 40 bp 3′ UTRVNTR in Saudi population. PCR technique was employed to detect polymorphisms of MAOA and DAT1 genes in a sample of 120 ADHD subjects and 160 controls. Alleles and genotypes frequencies for both of MAOA and DAT1 polymorphisms were compared among ADHD subjects against controls. Association between ADHD and alleles as well as genotypes for each studied polymorphisms was tested by odds ratio (OR) test and the magnitude of this association was estimated by 95 % confidence interval (95 % CI). A significant association was found between two MAOA genotypes 3/4 and 3/2 with ADHD ($P < 0.01$, OR $= 3, 4.9$) as a risk effect. No significant association was found with MAOA alleles. Among DAT1 polymorphisms two alleles (7 and 11 repeats) ($P < 0.01$, OR $= 2.5$ and 3.3) as well as two genotypes (11/11 and 11/7) ($P < 0.01$, OR $= 4, 3$) showed significant association with ADHD as a risk effect. On the contrary, 9 and 10 repeats revealed significant association as a protective effect as well as 10/10 and 10/9 genotypes. These findings support the hypothesis that some of the MAOA and DAT1 polymorphisms have a causative role in the development of ADHD in the Saudi population. Another polymorphism did not give rise to support this hypothesis. This is the first report investigated the association between MAOA and DAT1 polymorphism at molecular level in Saudi Arabia population as well as Arab world. Therefore further studies are needed to generalize obtained results at Saudi Arabia.

Keywords ADHD · Association · Polymorphisms · MAOA 30 bp-promoter VNTR · DAT1 40 bp 3′ UTRVNTR · Saudi Arabia

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Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most common childhood's behavioral disorders. It is characterized by marked inattention, hyperactivity and impulsiveness [1]. ADHD is affecting approximately 5 % of school-aged children; with boys predominating over girls at a ratio of 3:1 or more [2]. The prevalence of ADHD has been reported with great variations among different

studies, ranging from 2.2 to 17.8 %. These variations might be ascribed to population characteristics, methodological features, ethnic and cultural differences and/or diagnostic criteria [3]. The prevalence of combined ADHD in Saudi Arabia was found to be 16.4 %, where 12.4 % hyperactivity-impulsivity and 16.3 % inattention disorders [4]. These high percentages of ADHD incidence dictates the importance of research on ADHD in the Arabian populations, not only to assess the national prevalence in children and adolescents, but also to look at the differential burden and treatment of this disorder, which has high levels of mental co morbidities and high impact across the life span [5].

The etiology of ADHD is not clear nor completely understood [6], although it is a highly heritable disorder ($h^2 = 0.3$) [7] which refer to the direct genetic and environmental influences [8]. Molecular genetics and pharmacological studies suggest the involvement of dopaminergic, serotonergic and noradrenergic neurotransmitter systems in ADHD [9].

Monoamine Oxidase A (MAOA) is involved in the degradation of these neurotransmitters and it has been suggested as a strong candidate gene for ADHD [6]. Animal and human studies have implicated MAOA in impulsive and aggressive behavior [9]. MAOA gene is located on the X chromosome (Xp11.23–11.4). X chromosome genes are worthy candidates for studies in this disorder as ADHD is relatively infrequent among girls [2].

Genes code for enzymes and receptors involved in dopamine neurotransmitter pathways represent an attractive candidates, given that around 70 % of children with ADHD show a symptomatic improvement when treated with methylphenidate, a Psychostimulant which acts primarily to inhibit dopamine reuptake [10]. Dopamine Transporter 1 (DAT1) interacts directly with methylphenidate and mice homozygous for a DAT1 deletion exhibit hyper locomotion. DAT1 plays an important role in controlling blood levels of dopamine [11, 12]. Now it is well known that the genetic transmission is an important factor in ADHD etiology [13, 14]. No association studies of ADHD at molecular genetic levels were conducted among Saudi population. Therefore in the present study, the variable number of tandem repeat (VNTR) polymorphisms of the MAOA and Dopamine Transporter (DAT1) were selected as markers to study its possible association with ADHD in Saudi population.

Materials and methods

Subjects

Total 120 pure ADHD children between the ages 6–12 years from Pediatric Hospital and Prince Mansour Hospital at Taif city were included in the present study. In addition, 160 healthy age matched controls were recruited. All subjects were clinical referrals from hospital neurologists. Consensus diagnoses were made according to DSM-IV ADHD. Two scales, the abbreviated Conners Parent and Teacher Rating Scales [15] and the child Behavior Checklist, [16] were also employed. Non Saudi subjects as well as Autism cases were excluded from the study. No other neurological or behavioral disorders were identified.

Blood samples collection and genomic DNA extraction

Blood samples were collected into EDTA anticoagulation vacutainer tubes and transferred to laboratory of biotechnology and genetic engineering unit at Taif University. Oral consent was given to each of individual's parent and all institutional requirements were met. Genomic DNA was extracted from whole blood with the Qiagen Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). DNA was resuspended in 20 µl of ddH_2O and stored at −20 °C. Spectrophotometric determination of DNA concentration at A260 was done according to [17]. Total genomic DNA were electrophoresed on 1 % agarose gel (Bioshop Canada, Burlington, Ontario, Canada), stained with ethidium bromide (Bioshop Canada), visualized on a UV Transilluminator, BIORAD 2000.

MAOA genotyping

MAOA 30 bp-promoter VNTR polymorphic was characterized using PCR procedure according to [18] with the following primers :

MAO P1 5′-ACAGCCTGACCGTGGAGAAG-3′ and MAO P2 5′GAA CGGACGCTCCATTCGGA3′. The reaction mixture (20 µl) contained 200 mMdNTPs, 0.25 mM primers, 0.5 Unit Taq Gold (PerkinElmer Life Sciences, Boston, MA, USA) and 30 ng DNA. The amplification procedure included a 12 min prestart at 95 °C (for the hot start) and cycling conditions as follows: 95 °C for 35 s, 64 °C for 35 s and 72 °C for 50 s. A final extension at 72 °C for 5 min was employed.

DAT1 genotyping

Genotyping of DAT1 40-bp VNTR in the 3′-UTR was carried out according to [19] using a PCR procedure with the following primers as follows, upstream: 5′-TGTGGTGTA GGGAACGGCCTGAG-3′ and downstream: 5′-CTTCCT GGAGGTCACGGCTCAAGG-3′. Forty cycles were conducted consisting of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, and extension at 72 °C for 90 s. An initial denaturing step at 95 °C for 5 min and a last extension step at 72 °C for 7 min were also added. The 25 µl reactions

Table 1 Count and allele frequencies for MOA-A in ADHD and control group (100 %)

	2	3	4	5	Short (2, 3)	Long (4, 5)	Total
ADHD	50 (20.8)	80 (33.3)	70 (29.2)	40 (16.7)	130 (54.2)	110 (45.8)	240 (100)
Control	50 (15.6)	130 (40.6)	85 (26.6)	55 (17.2)	180 (56.3)	140 (43.8)	320 (100)
OR	1.4	0.7	1.1	1	0.9	1.1	
CI	09–2.1	0.5–1.0	0.8–1.6	0.6–1.5	0.7–1.3	0.8–1.5	
P value	0.1	0.07	0.5	0.9	0.6	0.6	

Table 2 Genotype frequencies for MAOA in ADHD and control group

	3/3	3/5	3/4	3/2	4/4	5/5	4/5	2/2	Total
ADHD	10 (8.3)	10 (8.3)	20 (16.7)	30 (25)	20 (16.7)	10 (8.3)	10 (8.3)	10 (8.3)	120 (100)
Control	50 (31.25)	10 (6.25)	10 (6.25)	10 (6.25)	30 (18.8)	15 (9.4)	15 (9.4)	20 (12.5)	160 (100)
OR	0.4	1.3	3	4.9	0.9	0.9	0.9	0.6	
CI	0.2–0.8	0.5–3.5	1.3–7.0	2.4–11.1	0.5–1.6	0.4–2.0	0.4–2.0	0.2–1.4	
P value	0.01	0.5	<0.01**	<0.01**	0.7	0.8	0.8	0.3	
		NS				NS	NS	NS	

Table 3 Count and allele frequencies for DAT1 in ADHD and control group (100 %)

	7-Repeat	9-Repeat	10-Repeat	11-Repeat	Total
ADHD	35 (14.6)	20 (8.33)	75 (31.25)	110 (45.8)	240
Control	20 (6.25)	65 (20.3)	175 (54.7)	60 (18.8)	320
OR	2.5	0.4	0.4	3.3	
CI	1.4–4.6	0.2–0.6	0.3–0.5	2.3–4.8	
P value	<0.01**	<0.01**	<0.01**	<0.01**	
		Significant protective	Significant protective		

were consisted of 100 ng genomic DNA, 10 Pmol of each primer, 20 mMdNTPs, 2 U of Taq polymerase, 10× Taq buffer, and distilled water.

Data analysis

After the amplification, the PCR reaction products were eletrophoresed with 100 bp ladder marker (Fermentas, Germany) on 10 × 14 cm 1.5 %-agarose gel (Bioshop; Canada) for 30 min using Tris–borate–EDTA Buffer. The gel was stained with 0.5 μg/ml of ethidium bromide (Bioshop; Canada).

All gels were visualized and documented using a Gene-Snap 4.00-Gene Genius Bio Imaging System (Syngene; Frederick, Maryland,USA). The digital image files were analyzed using Gene Tools software from Syngene.

Statistical analysis

Association of MAOA and DAT1 polymorphisms with ADHD was examined among cases and controls. In this case control study the allele frequencies of DAT1 and MAOA studied polymorphisms were estimated by direct counting and the difference in allele and genotype frequencies between the ADHD subjects and controls was tested using the odds ratio test. The magnitude of this association was estimated by 95 % confidence interval (95 % CI)

Results

In the present study 280 subjects were recruited (160 controls and 120 ADHD cases). MAOA and DAT1 polymorphisms were genotyped using PCR technique. Genotype and allele frequencies of DAT1 and MAOA genes were compared among a set of 160 controls and 120 ADHD subjects shown in Tables 1, 2, 3, 4 and Figs. 1, 2, 3, 4. Among MAOA polymorphisms five variants of a 30-bp repeat sequence have been reported: 2 (291 bp), 3 (321 bp), 4 (351 bp), 5 (381 bp) and 3.5 (336 bp). Four various repeat alleles belong to DAT1 40-bp VNTR in the 3′-UTR by size. These repeats were as follow, 7-repeat (360 bp), 9-repeat (440 bp), 10-repeat (480 bp), and 11-repeat (520 bp) were identified.

Table 4 Genotypes frequencies for DAT1 in ADHD and control group (100 %)

	10/10	10/9	11/11	11/7	11/10	10/7	Total
ADHD	10 (8.3)	20 (16.7)	35 (41.7)	20 (16.7)	20 (16.7)	15 (12.5)	120
Control	40 (25)	65 (40.6)	15 (9.4)	10 (6.25)	20 (12.5)	10 (6.25)	160
OR	0.3	0.3	4	3	1.4	2.1	
CI	0.1–0.6	0.2–0.5	2.1–7.9	1.4–7	0.7–2.8	0.9–5.1	
P value	<0.01**	<0.01**	<0.01**	0.01**	0.4	0.08	
	Significant protective	Significant protective			NS	NS	

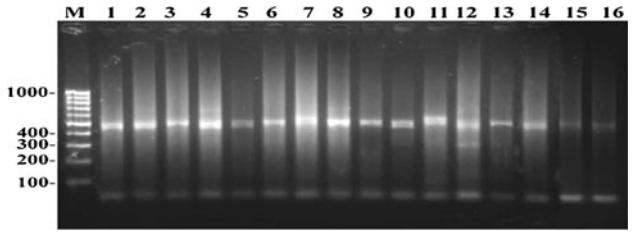

Fig. 1 Electrophoresis of PCR amplified MOA-A gene fragment among control subjects

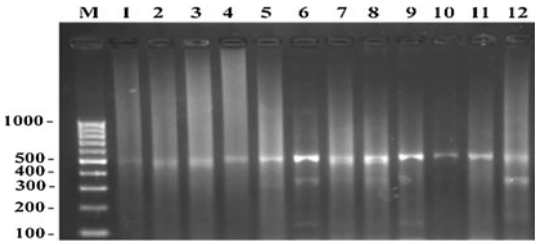

Fig. 2 Electrophoresis of PCR amplified MOA-A gene fragment among ADHD patients

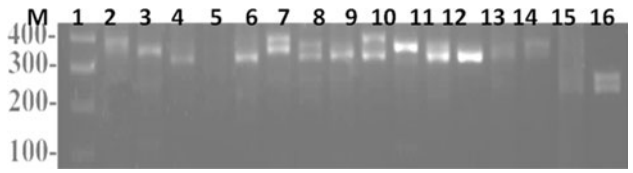

Fig. 3 Electrophoresis of PCR amplified DAT1 gene fragment among control subjects

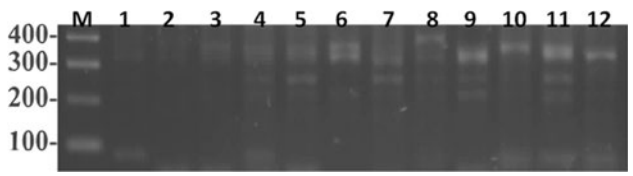

Fig. 4 Electrophoresis of PCR amplified DAT1 gene fragment among ADHD patients

Association between ADHD and polymorphisms of DAT1 and MAOA genes was investigated using odds ratio (OR) test. DAT1 polymorphisms analysis revealed identification of four alleles (7-repeat, 9-repeat, 10-repeat, 11-repeat) are shown in Table 3. Repeat 11 was the prevalent repeat (45.8) among ADHD subjects, while repeat 10 was the most frequent repeat among controls (54.7). Six genotypes were characterized (10/10, 10/9, 11/11, 11/7,11/10 and 10/7). Genotypes 11/11 and 10/9 are the most frequent genotypes among ADHD subjects (41.7 %) and controls (40.6 %) respectively. For MAOA 30 bp VNTR four alleles were detected (2, 3, 4 and 5) (Table 1). Allele 3 is the most common among ADHD subjects and controls. Eight genotypes (3/3, 3/5, 3/4, 3/2, 4/4, 5/5, 4/5and 2/2) were observed (Table 2). 3/2 genotype is the most frequent genotype among ADHD subjects (25 %), while 3/3 genotype was the most frequent one in controls (31.25 %). Among DAT1 allele and genotypes there were statistically strong association between 7 and 11 repeats and ADHD (OR: 2.5 and 3.3) respectively (Table 3). While, repeats 9 and 10 seems to have protective effects. 11/11 and 11/7 genotypes, revealed strong significant associated with ADHD (OR = 4 and 3) respectively (Table 4). On the other hand MAOA genotypes 3/4 and 3/2 showed highly significant association with ADHD (OR = 3 and 4.9) respectively, while no significant association was fond with MAOA alleles. It is also worth to mention that the *P* value for association with genotype 3 was 0.07 which is not very far from the level of significance.

Discussion

To the best of our knowledge this is the first report for investigation of these genetic polymorphisms in the etiology of ADHD in the Arab world. The highest allele frequency of DAT1 was for repeat 10, but without significant evidences of association with ADHD. These results are similar to the findings of [20, 21]. The results of the present study showed strong evidence of association between repeats 7 and 11 with ADHD. This results is in concordance with [22, 23]. In contrast to the other studies the 7 and 11 repeats have significant risk effect, whereas 9 and 10 repeat have a significant protective effect. These disagreements may be due to differences in population stratifications and/or methodologies [24] or differences in phenotype which reflect genetic heterogeneity [25, 26].

Several studies have been conducted to examine the association between some candidate gene and ADHD. Association between ADHD and the 10-repeat allele of a tandem repeat polymorphism located in the 3′ untranslated region of SLC6A3 was reported [27]. A replication study supports this association was described by [28, 29]. Several additional studies have been conducted to evaluate the association between DAT1 gene polymorphisms and ADHD among different ethnic groups via different research approaches (e.g. family-based association study method, case-control study method and both of methods). Some of these studies support the association [29–34] and others failed to confirm this association [20, 22, 23, 25, 35–40].

The results of MAOA gene polymorphism provide modest evidence for an association with children outcome of ADHD. These effects were significant for only two genotypes 3/3 and 3/2, but no evidence for association was observed for any given four alleles as well as short (2 + 3) and long (4 + 5) alleles. No association effect is consistent with previous reports [41–43]. Modest effect is in agreement with nominal effect reported by [44]. These contradictory reports about the role of both DAT1 and MAOA polymorphism in the etiology of ADHD may be due ethnicity and samples size. Therefore Multi centered future studies using genome wide scan and variable tandem repeat techniques with larger samples would be helpful for understanding the role of dopaminergic system at ADHD genetics [15].

Conclusion

In the present study, we investigated the association between ADHD and MAOA and DAT1 genes as markers in Saudi population. The findings of this study suggested that, there are different degrees of association. Some of polymorphisms showed significant association as a risk effect, while some of which did not exhibit any significant association. On the other hand some polymorphisms revealed strong association as a protective effect.

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