

## STUDY THE ASSOCIATION OF DAT1 GENE POLYMORPHISMS WITH ATTENTION DEFICIT HYPERACTIVITY DISORDER IN SAMPLE OF IRAQI PRIMARY SCHOOL CHILDREN

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### ABSTRACT

#### Summary

Attention Deficit Hyperactivity Disorder is a multifactorial disorder which results from combination of environmental and genetic factors. If children with ADHD are identified early and appropriate interventions are made the problem is likely to be mitigated.

The study was carried out to determine the frequencies of ADHD among Iraqi primary school pupils, and recognize the role of 3'UTR-VNTR of *DAT1* gene in ADHD.

The study sample included 1431 school pupils (777) males, and (654) females with age ranged between 8-12 years, selected from nine primary schools, at Baquba city. Revised version of RCBQ has been used as a means for identification of children with ADHD and prosocial (control). Genetic investigation were performed for Variable Number Tandem Repeats of 3'UTR *DAT1* polymorphisms and the sequencing carried out in BioScience company (Nottingham, UK), association between ADHD and alleles was tested by odd ratio, the magnitude of this association was estimated by 95% confidence interval.

The results revealed that 16.8% (241/1431) of those pupils have ADHD of combined type, males to females ratio was 2.2:1. This disorder was distributed highly among the age group 10-12 years. Out of 241 ADHD pupils, 118 (49%) suffer ADHD of combined type comorbid with aggressive symptoms, 65.3% (77/118) of them were males and 34.7% (41/118) were females. The VNTR ranged between 3 to 11 repeat, six alleles were identified 3, 7, 8, 9, 10 and 11R, while 4, 5, 6 R not found in this study. There was highly significant increase in the frequency of 10 R allele that found in 30%, 16% of ADHD and control groups respectively (OR=0.412, p<0.01), also there was highly significant increases in the frequency of the genotype 9/10 among ADHD group (OR=1, p<0.01), which showed significant association as a risk effect, while 9R and 10/11 genotype was highly significant increased in control group (OR=1, P<0.01), revealed significant association as a protective effect.

**Conclusion:** The frequency of ADHD symptoms among primary school children in Baquba city is high and appear in males more than females. This study showed that 10R and 9/10 genotype of *DAT1* 3'UTR significantly associated with ADHD while 11R and 10/11R genotype significantly associated with normal control children.

**KEYWORDS:** Gene Polymorphisms, Iraqi Primary School

## INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a problem of overactive, not being able to focus, unable control behavior, or a combination of these, despite being one of the most common childhood psychiatric disorder of neurodevelopmental type, children with ADHD usually have functional impairment across multiple settings including home, school, and peer relationships, these symptoms can make it difficult to succeed in school, get along with other children or finish tasks at home, and can continue through adolescence or adulthood <sup>(1)</sup>.

The exact cause of ADHD has not been determined, and the scientists are not sure what causes ADHD, it is believed to involve interaction between genetic and environment <sup>(2,3)</sup>.

Blum *et al.* (2008) <sup>(4)</sup> stated that, the cause or basis of ADHD is an impulse disorder with genetic components that results from imbalance of neurotransmitters. So it is a complex disorder having multiple causes including genetics as impacted by environment, might contribute to ADHD in addition to brain injuries, nutrition, is often blamed on bad parenting or bad attitude.

Neurotransmitter genes are among key candidates for evaluation complex behaviors and behavioral disorders, the neurotransmitter dopamine plays a major role in the frontal cortex, glucose metabolism and regional cerebral blood flow, these area is associated with important functions such as working memory and various aspects of cognitive <sup>(5,6)</sup>, it has a more protracted development than other areas of the brain with synaptogenesis continuing well in to middle childhood. The brain regions that are rich in dopamine activity, such as striatum, mid brain and frontal cortex, are involved in the disorder, strong functional connections between the striatum and different parts of the frontal cortex, given extensive evidence for frontal-

subcortical networks, dopamine genes affect processing in the frontal cortex and they acting primarily at the subcortical level <sup>(7)</sup>.

The dopamine transporter (DAT) is the primary mechanism for dopamine clearance from the synapse in the midbrain <sup>(8)</sup>.

Three polymorphisms in this gene have been studied: a highly polymorphic TaqI VNTR in intron 8 <sup>(9)</sup>, a biallelic TaqI RFLP (Taq 492) (10), and a 40-bp VNTR in the 3'- untranslated region, this final 40-bp VNTR has been the most studied for population variation <sup>(11)</sup>.

Over the past several years, there has been increasing interest in the potential involvement of *DAT1* gene in ADHD, it is the second gene suspected to be related to ADHD in the field of molecular biology, Cook and colleagues (1995) <sup>(12)</sup> found that the 3' untranslated region of *DAT1* has a variable number of tandem repeat (VNTR) polymorphism and ADHD was associated with 10 repeat allele, therefore, they first reported an association between ADHD and *DAT1* gene.

## SUBJECTS, MATERIALS AND METHODS SUBJECTS

The subjects selected for this study include one thousand four hundreds and thirty one (1431) Iraqi pupils, from nine primary schools from class 3<sup>th</sup>, 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> in Baquba city, randomly selected in a systematic random sample (N:

n, 87: 9 ). 777 were boys and 654 were girls, with age ranged between 8- 12 years. This study was carried out during a period from April 2012 to end of September 2014.

The demographical study was conducted on 1431 pupils, while the serological and genetic analysis was carried out on 100 ADHD pupils(65 were boys and 35 were girls) and 100 control group.

### Assessment and Scoring the Child ADHD Behaviour

Rutter Child Behaviour Questionnaire ( RCBQ ) was used as a scale for diagnosis of behavioral disorders having ADHD, versus prosocial behavior. Rutter Child Behaviour Questionnaire for completion by teacher in its original version was developed by Rutter at 1967, it consist of 59 items, 39 items for total difficulties ( conduct , Emotional, and hyperactivity ) and 20 items for prosocial trait <sup>(13)</sup>.

Special data sheet was designed for collection of two types of information for each child enrolled in this study.

- Information completed by the child family, about the child and his family, include personal and sociodemographic data, age, gender, class, school, years of scholastic failure, parental age, education, occupation, death, birth order, family size, crowding index. (number of family member number of rooms in the house ) polygamy, divorce and child working status.
- Information completed by the class teachers for school age children was score for each item either 0, 1 or 2 ( doesn't apply, applies somewhat and certainly applies ) ( Appendix-IV ). Child with a total score of nine or more (cut-off score at 50 th percentile), considered had ADHD.

### Genomic DNA Extraction

The genomic DNA isolated from two hundreds blood sample(5 ml.) collected in EDTA anticoagulant tubes from the two study groups, according to the protocol of Relia Prep <sup>TM</sup> BloodgDNAMiniprep System Kit.

The primers utilized in this study was provided by integrated DNA company Kopeckova *et al.*, 2008<sup>(14)</sup> (F-primer, 5'-TGT GGT GTA GGG AAC GGC CTG AG-3', R-primer,5'-GTTTCTT GGT CTG CGG TGG AGT CTG-, 3'). The variable number tandem repeat (VNTR ) of *DAT1* 3'-Untranslated region was detected using polymerase chain reaction in a total volume of 50 µl, the reaction component were PCR Go Taq Green Master Mix (promega- dATP, dGTP,dCTP ,dTTPs, MgCl<sub>2</sub> and reaction buffer (pH 8.5))25 µl.reverse primer and Forward primer 2 µl. DNA template 4µl,Nuclease free water17µl . with total volume50µl. The amplification was conducted using a TC-500 PCR according to the program as: Initial denaturation96(°C)for4 min.1Cycle, Denaturation94°C for30 sec., Annealing60°C-30 sec., Extension 72 °C -30 sec.(30 Cycles), and Final extension72°Cfor7 min. 1cycle.

The PCR products were separated on 2% agarose gel and were visualized by ethidium bromide staining.The PCR products of 200 samples of the amplified *DAT1* 3'UTR gene regions and primers were sending to Source BioScience Company (Nottingham, UK) for polymorphism and sequencing.

By use Blast program we compared the sequence of each sample in the sequence of *DAT1* gene which obtained from Ensemble Database. Sequence analysis was carried out at Asco Learning Center (Al-Harthyah,Baghdad-Iraq).

## RESULTS

The results revealed that 241(17%) of pupils had ADHD of combined type according to Rutter scale. In this study the age of pupils was allocated into two groups as, group one between 8-10 years and group two from 10- less than 12 years. The results revealed that 95/241(39.4%) of ADHD pupils were in first age group, while 146/241 (60.6%) in the second group Table-1.

Out of 241 ADHD pupils, 118 (49%) had both ADHD comorbid with aggressive signs, 77(65.3%) were males and 41(34.7%) were females, while 123(51%) had symptoms of ADHD without aggressive, included 87 males and 36 females. The main finding of this study is in the normal Baquba schools pupils based sample, about half of ADHD pupils are aggressive and males score higher ADHD comorbidity with aggression symptoms (according to the teachers) than females Table-2.

**Table 1: Distribution of ADHD Pupils According to Age and Gender**

School	Pupils No.	ADHD Pupils	Gender		Age Groups	
			Male	Female	8 -<10	10 -<12
1	243	35	26	9	15	20
2	216	36	26	10	15	21
3	159	27	20	7	10	17
4	177	27	17	10	10	17
5	171	29	20	9	10	19
6	138	23	13	10	8	15
7	114	20	12	8	8	12
8	114	19	10	9	9	10
9	168	241 16.8%	20	5	10	15
<b>Total</b>	<b>1431</b>	<b>25</b>	<b>164</b> 68.1%	<b>77</b> 31.9%	<b>95</b> 39.4%	<b>146</b> 60.6 %
			$X^2 = 2.31$ $P < 0.01$		$X^2 = 13.419$ $P < 0.01$	

**Table 2: Distribution of ADHD Pupils According to Comorbidity by Gender and Age Groups**

Gender	ADHD +Aggressive	Age Group		ADHD Without Aggressive	Age Group		Total No.
		8 -<10	10 -<12		8 -<10	10 -<12	
Male	7765.3 %	30	47	87	34	53	16468.1%
Female	4134.7%	18	23	36	13	23	7731.9%
<b>Total No.</b>	<b>11849 %</b>	<b>48</b>	<b>70</b>	<b>12351%</b>	<b>47</b>	<b>76</b>	<b>24116.8%</b>

According to the distribution of alleles and genotypes frequencies of the *DAT1* polymorphism in 3'untranslated region VNTR for both ADHD and control subjects as shown in table-3, the results found that sixalleles were identified among all ADHD pupils ranged from 3 to 11R, 3-R (200 bp), 7 R (360 bp), 8R (400 bp), 9 R(440 bp), 10 R(480 bp) and 11-repeats (520 bp). However, no four, five, or six-repeat alleles were observed in this sample of pupils (Figure-1). In the present study as determined by  $X^2$  test it was observed that the ten-repeat allele (10R) was the most frequent found in 30% of ADHD group and there were highly significant differences ( $X^2=18.672, p<0.001$ ) between ADHD and control groups. while the nine- repeat allele (9R) was the most common allele in control group 32% with a high significant differences ( $X^2=7.28, p<0.001$ ) compared to ADHD group.

Table 3: Allele Frequency of DAT1 3'UTR-VNTR in ADHD and Control Groups

Allele	ADHD		Control		X <sup>2</sup>	OR	C.I 95%
	No.	Percent%	No.	Percent%			
10R	30	30	16	16	16.62**	2.25**	(0.233-0.727)
10R/11R	2	2	14	14	4.439*	5.471*	(0.993-30.130)
11R	3	3	13	13	2.035	0.225	(0.026-1.955)
7R	6	6	-	-	0.989	0.909	(0.139-1.39)
3R	2	2	-	-	-	0.979	(0.140- 1.49)
3/10R	1	1	-	-	-	0.139	(0.29-2.9)
8R	15	15	9	9	4.286*	3.500*	(1.085-11.292)
9R	6	6	32	32	7.287**	2.125**	(1.283-3.518)
9R/10R®	35	35	16	16	0.89	1.00	( 0.39- 1.49)
<b>Total</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>			

® = Reference category. \*P<0.05 (S), \*\*P<0.01 (HS).

Additionally, it was found the most common genotypes in ADHD group 9/10R, while it is less common in control group found in 35%, 16% respectively (OR=1, CI=95%) as a risk effect, and 10/11R genotype more common in control group and their role as a protective effect(OR=5.471,CI=0.993-30.130).

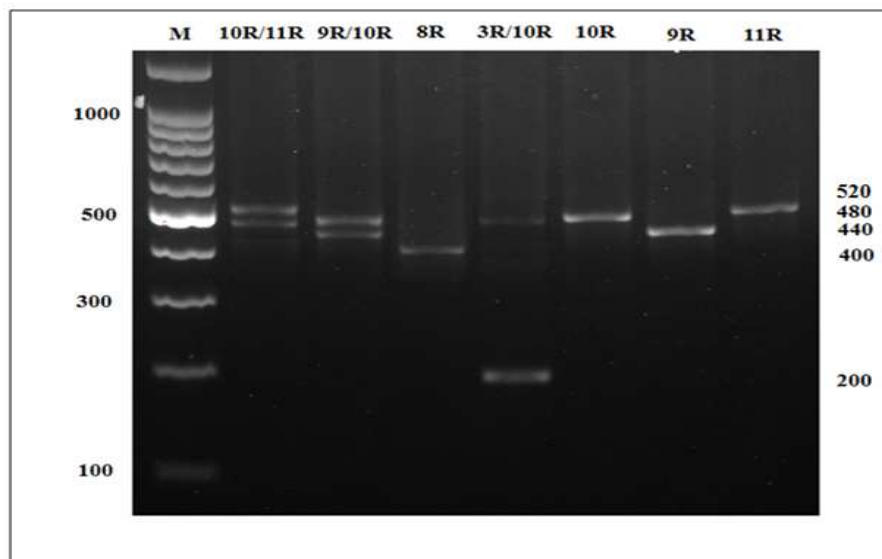


Figure 1: Ethidium Bromide Stained 2% Agarose Gel, Shows Allele Frequency of *DAT1* 3'UTR VNTR Primer Set, Show Heterozygous for the 9R&10R Allele, 10R&11R, at 100 Volts, in ADHD and Control Groups, M= DNA Marker (100 Bp), 3R=200 Bp, 8R=400 Bp, 9R=440 Bp,10R=480 Bp, 11R=520 Bp

The present study showed that the next most common allele is the eight-repeat (8R) occur in 15% of ADHD group, then the seven-repeat (7R) occur in 6% only in ADHD group while those repeats were not found in control group.

The present results showed that different forms of repeats ranged from three to eleven, and each repeat is different from the other in the number of the units that find inside it. with different sequence inside each unit, but all repeats start and end in the same sequences.

There are two levels of polymorphism in the VNTR of *DAT1* gene, the first is repeats number polymorphism (frequency) and the second is the sequence polymorphism among repeat units when examining the DNA sequences of alleles in our samples (figure-2 and 3).

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3R	P1	A	B	C	END														
7R	P1	A	A	D	E	F	D	C	END										
8R	P1	A	A	H	D	E	F	D	C	END									
9R	P1	A	A	G	H	D	E	F	D	C	END								
10R	P1	A	A	G	H	D	I	F	D	J	C	END							
11R	P1	A	A	G	H	D	D	I	F	D	J	C	END						

Figure 2: Allele and Repeat Frequency of *DAT1* 3'UTR- VNTR in ADHD and Control Groups

A	AGGAGCGTGCCTATCCCCGGACGCATGCAGGGCCCCCAG
B	AGGAGCGTGCCTATCCCCGGACGTATGCAGGGCCCCCAG
C	AGGAGCGTGTACTACCCAGGACGCATGCAGGGCCCCCAT
D	AGGAGCGTGTACTACCCAGGACGCATGCAGGGCCCCCAG
E	TGGAGCGTGTACTACCCAGGATGCATGCAGGGCCCCCAG
G	AGGAGCATGCCTATCCCTGGACGCATGCAGGGCCCCCAG
H	AGGAGCGTGTACTACCCAGAACGCATGCAGGGCCCCCAG
I	TGGAGCGTGTACTACCCAGGACGCATGCAGGGCCCCCAG
J	AGGAGCGTGTACTACCCAGGATGCATGCAGGGCCCCCAG

Figure 3: Sequences of Different Units of *DAT1* 3'UTR Region among ADHD and Control Groups

## DISCUSSIONS

The results of current study were similar to that stated by El-Tarraset *al.*(2012)<sup>(15)</sup> in Saudi Arabia who found that the prevalence of combined ADHD is 16.4%. but Polanczyket *al.*(2007)<sup>(16)</sup> found the rate of ADHD in America was higher than in Africa and in the middle east.

The center for disease control and prevention (CDC) (2013)<sup>(17)</sup> reported that the rate of ADHD diagnosis and treatment have increased in United kingdom and the United States, this can explained according to the Dalsgaard (2013)<sup>(18)</sup> who believed that the changes of the diagnostic criteria in 2013 with the release of DSM-IV increase the incidence of

people with ADHD especially in adults.

It was found that males were more commonly diagnosed with ADHD combined type (68.1%) than females (31.9%) with ratio 2.2:1.

This finding is consistent with the result of Al-Sharbatiet *al.*(2004 a and b)<sup>(19,20)</sup> who observed that the percentage of ADHD symptoms was 5.1% and 7.8% among Omani school girls and boys respectively.

American Psychiatric Association (2000)<sup>(21)</sup> stated that the ratio of boys versus girls with symptoms of ADHD ranges from 2-3:1 in community samples to 9:1 in clinical samples, while Hartung and Widiger (1998)<sup>(22)</sup> found equal gender distribution in investigation of adult ADHD.

The cause of the lower incidence of ADHD in girls might be related to having more commonly present with the inattentive subtype than males in the primary years aged 6-12 perhaps contributing to reduced rate of ADHD diagnosis among female<sup>(23)</sup>.

The distribution of ADHD pupils according to age showed that the highest percentage of ADHD signs were within the 10-12 years old, in contrast to studies of Biedermanet *al.*(2006) and Karamet *al.*(2009)<sup>(24,25)</sup> who were mentioned that the rate of ADHD lowered with increasing age, and these signs decrease in severity with age parallel to cerebral maturation, thus the disruptive behaviors in adolescence remain more pronounced in males, even though functional impairment continue well into adulthood.

There are several probabilities to why those children are aggressive, first of all the areas

Where they live suffered and still suffering from wars and shelling, as well as the environment was polluted.

The current results found that pupils with combined ADHD were highly comorbid with aggressive behavior came in agreement with finding of Eapenet *al.*(2009)<sup>(26)</sup> in United Arab Emirates children they have a range of psychiatric comorbidity with ADHD.

American Psychiatric Association (1994)<sup>(27)</sup> explained that, boys are more physically aggressive while girls are more likely to threaten by others without taking an action.

The variations in the prevalence rates as appeared from different researchers may be caused by the use of different cut off scores or the differences in the diagnostic criteria and the differences in the source of information that used which may contribute in this variation<sup>(28)</sup>.

The results of the present study came in accordance with the previous studies conducted in the world, that support the hypothesis that *DAT1* polymorphisms have a causative role in the development of ADHD<sup>(29,30)</sup>. In Jordan the results of study by Gharaibeh *al.* (2010)<sup>(31)</sup> supported the role of this gene in the development of ADHD, and found that 10R *DAT1* gene polymorphism were significantly associated with ADHD.

The findings of this study were clarified that 10R was associated with ADHD as a risk effect, in contrast to the results conducted in Saudi population by El-Tarraset *al.* (2012) who reported that 7 and 11 repeats (two alleles) as well as two genotypes 11/11 and 11/7 have significant association with ADHD as a risk effect, while 9 and 10 repeats revealed significant association as a protective effect.

Although Kang *et al.* (1999)<sup>(32)</sup> found that the ten-repeat allele is most common except for the middle eastern population in which the nine allele is most frequent, and stated that even among European populations this allele frequency was vary when used white or black U.S.A. populations and reported that this large variation in allele frequencies emphasizes the inadequacy of most past studies using the case–control design and importance of matching patient and control populations in future association studies.

It is in contradiction with study of wang *et al.* (2007)<sup>(33)</sup> didnt support that ADHD was in linkage disequilibrium with any polymorphism of the DAT1 40-bp VNTR locus, suggest that the transmission of 10- repeat allele polymorphism is not significantly associated with ADHD in Taiwan population, the absence of an association between ADHD and VNTR of *DAT1* 3' Untranslated region alsowas observed in studies carried out in Brazil and Norway<sup>(34,35)</sup>.

The variation of allele distribution or discrepancy may due to the complexity of ADHD, the differences in the genetic background of the studied populations and the impact of environmental factors<sup>(36)</sup>.

Other explanation reported by Nakamura *et al.* (1998)<sup>(37)</sup> because the location of VNTR in the 3' - non coding region may function as transcriptional regulators, and the allelic variants can not result in structural differences in human DAT protein.

Waldman and Gizer (2006)<sup>(38)</sup> stated that the dopamine transporter1 (*DAT1*) gene plays an important role in controlling blood levels of dopamine, but it is not clear how the polymorphism of VNTR could affect *DAT1* function in the human brain, while Bedard *et al.* (2010)<sup>(39)</sup> found that *DAT1* 3'-UTR polymorphism influences neural activity in a corticostriatal circuit implicated in the pathophysiology of ADHD.

## CONCLUSIONS

- Emphasis should be put on specialized training courses under direct medical supervision for all teachers and social worker to use this scale for periodic evaluation program to assess child's behaviour throughout the educational year.
- It is important to have an educational psychologist as a constant staff in all primary schools as for teachers to be a ware of this behavior in order to achieve early diagnosis counseling and behavior rectification.
- Attract the attention of the parents about this problem which is increasing contiuously ,to enable them to make care of their children ( school boys ) during the last years with increased the witness violence.
- Can conduct more extensive analytical studies about this critical and important field with the ultimate aim of obtaining more valid information to overcome the prob
- Future studies will need to type each of genes related with ADHD, and measure each environmental risk factor in a single well characterized sample of individuals with ADHD to fully understand the complex etiology of ADHD.



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