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Research Article

Association of dopamine D4 receptor gene variants with autism

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ABSTRACT

Background: Autism spectrum disorder (ASD) is a heritable neurodevelopmental disorder with poorly understood and complex etiology. The central dopaminergic system is strongly implicated in ASD pathogenesis. Genes encoding various elements of this system have been linked to ASD. This study aimed to estimate the distribution frequency of dopamine D4 receptor-exon III repeat region polymorphic genotypes among Egyptian children with autism.
Methods: This case-control study included 178 children with autism (mean age 4.46±1.72 years) (118 males and 60 females) and a normal control group (n=128) of matching age and gender. Assessments by DSMIV- TR criteria, Stanford-Binet intelligence scale and childhood autism rating scale (CARS) were done. Assay for DRD4 48 bp VNTR genotypes was performed on amplified DNA by RFLP-PCR.
Results: The 4/4 allele had the highest frequency among both autistic (39.32%) and control children (62.5%), with no significant difference between them. The 7/7 allele had also a high frequency (33.7%) among autistic patients, which was significantly different ($p<0.05$) from the control group (12.5%) Furthermore, 70% of the patients carrying the 7/7 allele had the lowest IQ scores (58.5±6.5).
Conclusions: There is a strong evidence that the DRD4 7/7 allele might be a risk factor for autism.

Keywords: Autism, Dopamine D4 receptor, Gene polymorphism, Egyptian children

INTRODUCTION

Autism is a complex disorder that involves the interactions among multiple organs, including the brain, immune, gastrointestinal and other systems.[1-3] Recognized for its 'spectrum' nature, autism spectrum disorder (ASD) is a serious neurodevelopmental illness that affects approximately 1–2% of the general population, and has a significant societal and mental health impact.[4,5] Many candidate genes have been identified in autism. Although a strong genetic component has repeatedly been identified in ASD, the genetic cause of ASD is still unknown for the majority of ASD cases.[6] Therefore, understanding the genetic determinants and associated molecular pathways of ASD may enable its better treatment and prevention.[1-3,7]

From a historical perspective, several lines of evidence from early clinical studies on dopaminergic modulation implicated dopamine deficits in ASD and autism.[8,9]

The neurotransmitter dopamine (DA) plays an important role in the central nervous system by regulating a variety of functions, including motor activity, motivation, attention, and reward. Disrupted DA function is implicated in a number of neuropsychiatric disorders, including bipolar disorder, schizophrenia, attention-deficit hyperactivity disorder (ADHD) and, ASD.[10]

In 1979, Kebabian and Calne found that dopamine exerts its effects by binding to two receptors, known as the D1 and D2 receptors. These two receptors exert their biological actions by coupling to and activating different G protein complexes. The D1 receptor interacts with the Gs complex to activate adenylyl cyclase, whereas the D2 interacts with GI to inhibit cAMP production.[11]

The application of homology screening techniques led to the characterization of three new dopamine receptors: D3, D4, and D5. In their putative transmembrane domains, the D1 and D5 receptors are 79% identical but are only 40–45% identical to the D2, D3, and D4 receptors. Conversely, the D2, D3, and D4 receptors are between 75% and 51% identical to each other, the first indication that the five receptors can be divided into the D1-like and D2-like receptor subfamilies. The existence of different variants of the human D4 receptor has been demonstrated. These variants differ in the number of 48 base-pair repeats contained in their putative third cytoplasmic loop and they have been detected in the genomes of different individuals, showing that a genetic polymorphism is responsible for the generation of the D4 receptor variants. These repeats are not present in the rat gene, making the polymorphism specific to humans. These variants can behave differently with respect to the mechanism of ligand recognition.[12] Dopamine receptors are implicated in many biological (mainly neurological) processes, including cognition, memory, learning, and motor control, as well as modulation of neuroendocrine signaling, and thus are connected to many psychiatric and neurological disorders, including ADHD and autism.[13]

Aim of the study

Despite the large number of studies in the field of autism and ASD, a study on the dopamine D4 receptor gene polymorphisms and it's association with autism in Egypt is still lacking; hence, the aim of the present work was to study the possible association between polymorphic VNTRs of the DRD4 gene and increased risk for autism.

Patients

This work was done after taking acceptance of all patients' and controls' parents to share in the study as well as acceptance of ethics committee of the University.

Table 1: Descriptive data of the patients group.

Sex	**No. (%)**
Male	118 (66.29)
Female	60 (33.7)
Age Range	2.00-11.00
Mean±*SD*	4.46 ±1.72
CARS	Mean± SD = 37.68± 5.3
IQ	Mean± SD = 67.32± 7.63
DSM	Mean± SD = 7.14± 1.03

The work has also been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. This case control study enrolled 178 child patients with autism diagnosed by DSM-IV-TR criteria.[14] Patients (Table 1) were recruited from the Psychiatric Clinic, Pediatric Hospital, Ain Shams University. They were 118 males (66.29%) and 60 females (33.7%). Their ages ranged from 2 to 11 years (mean age 4.46± 1.72 years). The control group included 128 healthy children [77 males (60.15%) and 51 females (39.84%)]. Their ages ranged from 3 to 8 years (mean 4.9± 1.58 years). The control children were referred to the psychiatric clinic to exclude the presence of autism spectrum disorders (ASDs).

METHODS

1. Confirmation of diagnosis:
   Confirmation of diagnosis was performed using a DSM-IVTR criteria questionnaire of autism, i.e. impairments of language, social skills, and restricted stereotyped interest or activity.14 Assessment of mental age was done using the Stanford-Binet intelligence scale (2003) to calculate the intelligence quotient (IQ). 15 Subnormal intellectual functions are diagnosed when IQ is below 70. Assessment of severity of autistic symptoms using the childhood autism rating scale 16 which rates the child on a scale from one to four in each of fifteen areas (relating to people, emotional response, imitation, body use, object use, listening response, fear or nervousness, verbal communication, non-verbal communication, activity level, and consistency of intellectual response, adaptation to change, visual response, taste, smell, touch response and general impression) was done.
2. DRD4 48 bp VNTR genotyping:
   DNA was extracted from whole blood using a QIAamp Blood mini-prep Kit (QIAGEN, Germany) according to manufacturer' s instructions. The DRD4-exon III repeat region was genotyped by a PCR-based restriction fragment length polymorphism analysis (RFLP). The oligonucleotide primers used were: forward 5'-ACCACCACCGGCAGGACCCTCATGGCCTTGC GCTC-3' and reverse 5'-CTTCCTACCCTGCCCGCTCATGCTGCTGCTCT ACTGG-3'. 17 The previously mentioned primers were used to generate the DRD4-exon III polymorphic region [2–10variable repeat units (R), 1 unit=48 bp]. DNA samples (500 ng) were amplified in the presence of 10 pmol of each primer, 1x PFU PCR buffer, 0.4 mM each dNTP- dATP, dTTP, dCTP and dGTP), 200 mM 7-Deaza-dGTP, 5% DMSO, and 2U PFU Taq. The PCR protocol was performed on thermal cycler HVD ™, Austria as follows: A denaturation step at 94ºC for 2 minutes, followed by 35 cycles consisting of 98ºC for 45 seconds, 55ºC for 45 seconds, and 72ºC for 1 min 30

seconds, with a final extension step at 72ºC for 10 minutes. Analysis of the amplified products was done on agarose gel electrophoresis 1.8% stained with ethidium bromide in 1x Tris-EDTA (ethylenediamine tetra acetic acid)-Borate buffer (TBE) against 100 bp ladder molecular weight marker (Fermentas™, Finland) to detect the corresponding amplified fragments (figure 1). The PCR fragments were all documented by Gel Documentation System and Software for DNA analysis (In Genius Syngene™ – UK) using 100 bp molecular weight ladder (Fermentas™, Finland) for confirmation of proper PCR product length. The distribution of polymorphisms, genotypes (repeat numbers- R) and allele frequencies were all statistically compared in all patients versus healthy controls.

3. Statistical analysis:
Statistical analyses were carried out with SPSS 20.0 software for Windows. Comparisons of the allele and genotype frequencies between groups were assessed using non-parametric $\chi 2$ test. The criterion for significance was set at $p< 0.05$ for all the tests.

RESULTS

Genotyping for DRD4- 48 bp VNTR (Table 2) revealed that the 4/4 and 7/7 alleles are predominating among the patients' group, with frequencies of 39.32% and 33.7%, respectively (Figure 1; A, B and C).

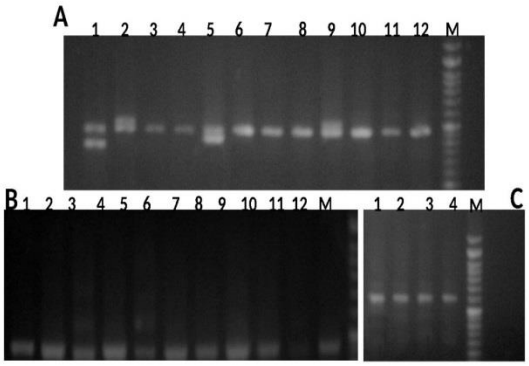

Figure (1): PCR analysis of exon III 48bp VNTR of the dopamine receptor 4 gene (DRD4)

M: Marker 50 bp
Lane 1-A, 4-B and 11-B : 2/4 repeat type (379 / 475 bp)
Lane 1-B: 3/3 repeat type (427 bp)
Lane 5-A: 3/4 repeat type (427 / 475 bp)
Lane (3, 4, 6, 7, 8, 10, 11, 12)-A, (2, 3, 5, 6, 7, 8, 9, 10, 12)- B: 4/4 repeat type (475 bp)
Lane 2-A and 9-A : 4/5 repeat type (475/523 bp)
Lane (1, 2, 3, 4)- C: 7/7 repeat type (619 bp)

Figure 1: PCR analysis of exon III.

Table 2: Comparison between patients and controls regarding genotype frequency distribution.

Genotype	Patients		Control		*p*-value
	No.	%	No.	%	
2/4	24	13.48	18	14.06	NS
3/4	12	6.74	8	6.25	NS
4/4	70	39.32	80	62.5	NS
4/5	8	4.5	4	3.12	NS
7/7	60	33.7	16	12.5	$p<0.05^*$
3/3	4	2.25	2	1.56	NS
Total	178	100	128	100	

* $p<0.05$ is considered statistically significant

Table 3: Distribution of DRD4 polymorphic genotypes among different IQ levels.

Genotype	2/4		3/4		4/4		4/5		7/7		3/3	
IQ	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Low average (80-89)	-	-	-	-	-	-	-	-	-	-	-	-
Borderline delayed (70-79)	14	58.33	-	-	38	54.29	-	-	8	13.33	-	-
Mildly delayed (55-69)	10	41.66	12	100	30	42.86	8	100	10	16.66	4	100
Moderately delayed (40-54)	-	-	-	-	2	2.86	-	-	42	70	-	-
IQ (mean±SD	67.67 ±9.3		62.5 ±3.5		72.33 ±7.4*		66 ±7.07		58.5 ±6.5*		63.2 ±2.5	

Comparison between patients and controls regarding DRD4- 48 bp VNTR genotype frequency (Table 2) revealed no significant difference between the two groups, except for the 7/7 allele, which showed a

statistically significant difference between its frequency in the patient group and controls (p<0.05).

DISCUSSION

Autism is a complex neuro-developmental disorder that is increasingly being recognized as a public health issue. Knowledge of genes of major effects allows identification of specific diagnostic biological markers. In the absence of clear definite objectives of clinical characteristics, the identification of these peripheral biomarkers in ADHD would be highly relevant for the diagnostic process. Identifying risky individuals at a younger age would allow implementation of treatments sooner to decrease the severity of ADHD symptoms or even to prevent future symptoms.[18]

To our knowledge, no studies have been published on the linkage between DRD4 gene polymorphism and increased risk for autism in Egyptian autistic children.

In this study, 178 Egyptian children diagnosed with autism as well as 128 healthy control children, were genotyped for the DRD4 48 bp VNTR polymorphism. The 4/4 genotype was significantly (p<0.05) more prevalent among controls (62.5%), compared to autistic patients (39.32%). Meanwhile, the 7/7 genotype was significantly (p<0.05) more prevalent in autistic patients (33.7%) compared to controls (12.5%).

The distribution frequency of the other DRD4 genotypes 2/4, 3/4, 4/5, and 3/3 among autistic patients was as follows: 13.48%, 6.74%, 4.5% and 2.25%, respectively. On the other hand, the distribution frequency of the same DRD4 genotypes 2/4, 3/4, 4/5, and 3/3 among control children was as follows: 14.06%, 6.25%, 3.12, and 1.56%, with no significant differences between the two groups.

Comparing IQ scores of autistic children among each DRD4 genotype revealed that 70% of the patients carrying the 7/7 genotype, were classified as moderately delayed, while only 13.3% of the patients carrying the same genotype were classified as borderline delayed. Meanwhile, for the 4/4 genotype, 54.29% of the patients were classified as borderline delayed, while only 2.86 % were classified as moderately delayed. There was a statistically significant difference (p< 0.05) in mean IQ score between the patients carrying the 4/4 genotype (72.33 ±7.4) and those carrying the 7/7 genotype (58.5 ± 6.5).

Previous analysis of a large worldwide sample of autistic patients have shown similar DRD4 distribution frequency, where the four repeat D4 receptor allele was the most common (~65%), followed by the seven-repeat (~25%) and two-repeat forms (~5%), with large differences between ethnic groups. Furthermore, Angela et al.[19], have found that the DRD4 7-repeat allele is associated with clinically elevated autistic traits (high SRS score) among individuals with ADHD. Authors have also found that forms of ADHD that are associated with the DRD4 7-repeat allele might generally be characterized by an increased level of autistic social impairment. Moreover, Faraone and Khan[20], have also revealed that the presence of the seven repeat allele is associated with a three- to fivefold higher risk of ADHD, and contributes to lower IQ scores.[21]

The current finding about the 7/7 allele can be understood in light of the findings of Ding et al.[22] that the 7-repeat allele encodes a receptor with lower affinity for dopamine. In vitro studies indicate that the sensitivity of the 7R allele is half that of the 2R and 4R variants.[22]

In terms of binding of adenyl cyclase, the DRD4 7-repeat receptor was found to be two to three times less potent than 4-repeat or 2-repeat receptors. In vitro studies demonstrated that the 7-repeat allele of the 48-bp repeat polymorphism leads to a blunted response to dopamine.[23] In accordance, the 7R allele was found to be associated with various psychiatric disorders including ADHD, dependences, pathological gambling, alcoholism and drug dependence.[24] Several studies also described associations with schizophrenia and autism.[25-27]

There is evidence that the seven-repeat allele arose by a relatively recent mutational event about 50,000 years ago, and that it exhibits positive selection.[28] The seven repeat allele was also found to be associated with increased novelty-seeking behaviors, and the level of attention-associated gamma synchrony is greater in subjects with the seven-repeat allele, as compared to two or four repeats.[29]

In a pilot study by Shahin *et al.*[30] concerning DRD4 gene polymorphisms as risk factor in Egyptian children with ADHD, the authors have found that there is a significant association between the possession of the four repeat allele with the children with ADHD alone than the ADHD with autistic features, whereas 7R was only detected in the ADHD without autistic features and constituted 7.89%.

CONCLUSION

There is a strong evidence that the DRD4 -48bp VNTR 7/7 allele might be associated with autism in Egyptian children. However, further studies, with larger samples and varying age groups are needed to confirm the relationship between DRD4 48bp VNTR polymorphisms and increased risk for autism.

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