***DRD4* and *DAT1* VNTR Genotyping in Children with Attention Deficit Hyperactivity Disorder**

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Abstract The dopamine receptor-D4 and the dopamine transporter have been investigated for their role in attention deficit hyperactivity disorder (ADHD) in children. Reports of their genetic association with ADHD have shown mixed results. The aim of the study was to evaluate the association of variable number tandem repeats (VNTRs) of the *DRD4* and *DAT1* genes with ADHD in children. A pilot 1:1 case control study, with 44 clinically confirmed ADHD cases and 44 age/gender matched healthy controls, was conducted at a tertiary care centre in Mumbai. Variable number tandem repeats of *DRD4* exon 3, *DAT1* intron 8 and 3'UTR were genotyped by PCR-AGE. Several allele repeats of the genes were observed in the screened subjects. Statistical significance was observed for the 10R/10R genotype of the *DAT1* 3'UTR VNTR between cases and controls.

Keywords ADHD · *DRD4* · *DAT1* · VNTR

Abbreviations

ADHD	Attention-deficit/hyperactivity disorder
<i>DRD4</i>	Dopamine receptor D4
<i>DAT1</i>	Dopamine transporter
VNTR	Variable number tandem repeats
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
UTR	Untranslated region
R	Repeat

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a childhood onset-neurobehavioral disorder defined by persistent and maladaptive symptoms of hyperactivity/impulsivity and inattention. Based on the symptoms, the three presentations of ADHD include predominantly hyperactive-impulsive, predominantly inattentive and combined [1]. The worldwide prevalence of ADHD is 3.4 % (95 % CI: 2.6–4.5) [2].

ADHD is a complex, clinically heterogeneous disorder depending on age, gender and presence of co-morbidities [3, 4]. It results in impaired functioning in multiple settings with severe impulsiveness, lack of concentration and motor hyperactivity as its core clinical features. ADHD is often accompanied by other psychiatric disorders and learning difficulties, with problems of antisocial behavior and substance abuse [5]. Neuroimaging assays have consistently implicated abnormalities of the prefrontal cortex and basal ganglia in the pathophysiology of ADHD [6].

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ADHD has a mean heritability estimate of 76 %; understanding the genetic factors that increase risk of ADHD can help elucidate its pathophysiology [5]. The *DRD4* gene encoding for the dopamine receptor-D4 and the *DAT1* gene encoding for dopamine transporter from the catecholaminergic system are most frequently investigated for association with ADHD. Candidate gene studies have focused on the 48-bp variable number tandem repeat (VNTR) in the exon 3 of the *DRD4*, a 40-bp VNTR in the 3'UTR and a 30-bp VNTR in the intron 8 of the *DAT1* [7].

Various ethnic groups have reported different allele frequencies of these VNTRs. Replication studies have found mixed results of the genetic association across various population groups [7]. Thus, the pilot study was undertaken to evaluate the correlation of *DRD4* and *DAT1* gene variants with ADHD, in Indian children from a tertiary care setting in Mumbai.

Materials and Methods

Sample Selection

Children from the age of 5–15 years, undertaking a psychiatric evaluation at a mental health clinic at the outpatient department of a tertiary hospital in Mumbai were recruited for the study. The subjects were sampled from a heterogeneous population. In all, 44 cases (6 girls, 38 boys) with a definitive diagnosis of ADHD as per the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) guidelines were included in the study [1]. Subjects with a known history of any other mental disorder(s) were excluded from the study. The controls were age and gender matched children with no history of any mental disorder, visiting the pediatric clinic. Informed consent from the parents of the subjects and research assent from children above the age of eight was collected. Patient information sheet was provided to all the study participants. The study was approved by the institutional review board of the hospital.

Genotyping

Venous blood samples were collected in BD Vacutainer® EDTA tubes. DNA was extracted by the modified Miller et al. method [8] and subjected to PCR amplification. The PCR cocktail for both the VNTRs of *DAT1* included 1X PCR Buffer, 200 mM dNTPs, 20 pM primers, 1.5 mM MgCl₂, 2.5 U Taq Polymerase (Thermo Scientific). The *DRD4* Exon 3 VNTR was amplified using 1X Hot Star Taq Buffer (containing 15 mM MgCl₂), 200 mM dNTPs, 1X Q solution, 10 pM primers and 2.5 U HotStar Taq polymerase (Qiagen). The primers (Sigma Aldrich) were designed as

per literature [9–11] with modifications. The amplicons were visualized by on a 3 % agarose gel with a 100 bp DNA ladder (Thermo Scientific). Genotyping was confirmed by automated DNA sequencing of representative samples from Chromous Biotech, Bengaluru, India.

Statistical Analysis

Statistical Analysis was performed by Epi Info Software (Epi Info™ 3.5.3). Chi square test was used to compare the VNTR frequencies between cases and controls.

Results and Discussion

PCR-VNTR genotyping of *DRD4* exon 3 VNTR, *DAT1* intron 8 and 3'UTR VNTRs was done in the 44 cases and 44 controls.

The patient demographics of the subjects are as described. All the patients showed a minimum 6 of the 9 inattentive and/or hyperactive/impulsive symptoms for more than 6 months at two or more settings. There was an equal number of ADHD probands in the age bracket of 5–9 and 10–15 years ($n = 22$). There were more boys ($n = 38$) than girls ($n = 6$) among the test subjects. Majority of the patients were diagnosed with the combined subtype ($n = 32$). Some of the patients had a comorbid learning disorder. Several patients were on remedial therapy. Some were prescribed atomoxetine or methylphenidate as treatment [12]. The subjects came from various language and ethnic backgrounds.

Genotyping results are as mentioned in the Table 1. The VNTR alleles of the *DRD4* gene exon 3 found in our study group include 2-repeat (R), 3R, 4R, 5R, 7R alleles. The 4R allele had the highest frequency in both cases and controls. Meta-analysis studies in Caucasian cohorts have shown a significant association ($p < 0.00001$) between ADHD and the 7R allele of the *DRD4* exon 3 [13]. In contrast, the shorter 2R allele was reported to be associated with ADHD in a Chinese Han study, with a complete absence of any higher repeats [14]. In our study, genotypes of both the longer repeat 7R/7R and the shorter repeat 2R/2R were rare. None of the alleles were significantly associated with ADHD cases. However, in a study on subjects from Eastern India, a preferential transmission of 7R allele was observed in the ADHD probands [11]. These results indicate a possible difference in the allele frequencies of the *DRD4* VNTR 's across different ethnic groups.

For the *DAT1* gene, the VNTRs in two regions were studied. 6R, 9R, 10R, and 11R alleles were found in the 3'UTR region and 5R, 6R, 7R, 12R, and 13R alleles were found in the intron 8 region. The 10R allele of the 3'UTR

Table 1 Genotyping of *DRD4* and *DAT1* VNTRs

<i>DRD4</i> exon 3	Cases	Controls	<i>DAT1</i> 3'UTR	Cases	Controls	<i>DAT1</i> intron 8	Cases	Controls
4R/4R	34	30	10R/11R	1	0	13R/6R	2	0
4R/2R	6	7	10R/10R	38	26	13R/5R	0	1
4R/3R	0	3	10R/9R	4	17	12R/6R	1	1
3R/3R	1	0	6R/6R	1	1	7R/6R	0	1
2R/2R	2	2				6R/6R	28	26
4R/5R	1	0				6R/5R	11	14
2R/5R	0	1				5R/5R	2	1
7R/7R	0	1						

and the 6R and 5R alleles of the intron 8 VNTR were the most common alleles among cases and controls. The frequency of the other alleles was low. The frequency of the 10R/10R genotype of the *DAT1* 3'UTR VNTR region was found to be statistically significant between cases and controls ($\chi^2 = 8.25$, $p = 0.0041$, OR 4.31, 95 % CI: 1.53–12.53). This finding is similar to previous Caucasian studies including a meta-analysis which suggests an association ($p = 0.002$) of the *DAT1* 3'UTR 10R allele with ADHD [13]. This allele has been associated with increased expression of the *DAT1* gene [15].

Additionally, the 10R/9R genotype of the *DAT1* gene was seen in more controls than cases in the present study. ($\chi^2 = 9.01$, $p = 0.0027$, OR 0.1588, 95 % CI: 0.0481–0.5240). None of the alleles of intron 8 VNTR of the *DAT1* gene showed a significant association with ADHD. However, in the study on subjects from Eastern India, the 9R allele of the *DAT1* 3'UTR was found to be implicated in ADHD cases while the 5R allele of the intron 8 of the *DAT1* gene occurred higher in cases [11]. These differences in the *DAT1* VNTR allele frequencies, may be due to ethnic differences, as the present study included subjects from a mixed population group.

To conclude, the results of our pilot study implicate the 10R/10R genotype of the *DAT1* gene 3'UTR to be statistically significant in ADHD cases and the 10R/9R genotype in the controls. Several studies have also implicated this 3'UTR VNTR of the *DAT1* gene in the pharmacogenomics of ADHD. However, in a recent meta-analysis, the effect of the VNTR on response to methylphenidate treatment was not found to be statistically significant. The role of other genetic polymorphisms in treatment response across ADHD subjects, needs to be further studied [16].

The limitation of this study was the small sample size, and 32 of the 44 cases were of the combined subtype. A larger study with an equal number of combined, inattentive and hyperactive subtype patients might provide a better understanding between genotype and disease subtype. Correlation between genotype and response to ADHD treatment also needs to be evaluated. This can lead to

improved diagnosis, better management of symptoms and appropriate medication in subjects with ADHD.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no potential conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent from all the parents of the study subjects and research assent from children above the age of eight was obtained.

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