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Sequence Variants of the *DRD4* Gene in Autism: Further Evidence That Rare *DRD4* 7R Haplotypes Are ADHD Specific

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A high prevalence of rare dopamine receptor D4 (*DRD4*) alleles in children diagnosed with attention-deficit hyperactivity disorder (ADHD) has been reported [Grady et al., 2003]. In this prior study, extensive resequencing/haplotype data of the *DRD4* locus was used to suggest that population stratification was not the explanation for the high prevalence of rare alleles. In the current study, DNA resequencing/haplotyping was conducted on 136 *DRD4* alleles obtained from autism probands, collected from the same geographic population as the prior ADHD probands (Orange County, CA). A number of studies have suggested that the susceptibility genes underlying these two disorders might partially overlap. Rare *DRD4* variants were not uncovered in this autism sample beyond that expected by chance. These results suggest strongly that the high prevalence of rare *DRD4* alleles in ADHD probands is due to ascertainment of the sample by diagnosis of ADHD.

KEY WORDS: attention-deficit hyperactivity disorder; dopamine receptor D4; VNTR; DNA resequencing

INTRODUCTION

Autism is a relatively common neurodevelopmental disorder with an incidence of approximately 0.2%. Symptoms are apparent during the first three years of life, and comprise limited verbal communication, a lack of social reciprocity, and repetitive and stereotyped behaviors [Bristol et al., 1996]. Twin studies have indicated a strong genetic component for autism, however genome scans (to date) have failed to identify genes of major effect [Gutknecht, 2001].

A common interpretation of these negative genome scan results is that multiple rare autism susceptibility genes/alleles exist. Alternatively, however, these negative results may merely reflect insufficient power to detect common predisposing alleles in the examined populations. This "Common variant-common disorder (CVCD)" hypothesis [Risch and Merikangas, 1996; Zwick et al., 2000] proposes that common disorders are related to common variants, and hence their relative increase in frequency in affected individuals will be modest (less than 2–4 fold). If this CVCD model is correct, then only a few predisposing alleles, acting in combination, could account for much of the genetic risk [Grady et al., 2003]. Such alleles would not be detected with genome scans of current size [Risch and Merikangas, 1996]. A candidate gene approach, in contrast, has sufficient power to detect a common allele in the chosen gene if it is associated with the disorder being investigated.

Attention-deficit hyperactivity disorder (ADHD) is 25 times more common than autism (approximately 5% incidence), making it the most prevalent disorder of childhood [American Psychiatric Association DSM-IV, 1994; MTA Cooperative Group, 1999]. It is defined by symptoms of developmentally inappropriate inattention, impulsivity, and hyperactivity [Swanson et al., 2000; Grady et al., 2003]. The efficacy of a dopamine agonist drug, methylphenidate, in the treatment of ADHD has suggested that genes in the dopamine pathway may be involved in the disorder's etiology [Swanson et al., 2000]. Using a candidate gene approach, the association of a variant of one of these genes (*DRD4* 7R) with ADHD is now one of the most reproduced in complex behavioral disorders [Faraone et al., 2001; Grady et al., 2003]. Recently, we presented DNA resequencing/haplotype data of the *DRD4* locus indicating that the 7R allele is of recent origin, and has likely increased in frequency by positive selection [Ding et al., 2002; Wang et al., 2004]. We further showed by direct DNA resequencing/haplotyping that over 10% of ADHD probands have novel rare *DRD4* alleles, mostly 7R allele derivatives [Grady et al., 2003].

Some studies have suggested that the susceptibility genes underlying these two disorders (autism and ADHD) might partially overlap [Smalley et al., 2002; Yamagata et al., 2002; Ogdie et al., 2003]. However, by definition, DSM-IV diagnosed autism and ADHD exhibit a spectrum of behaviors that are in some ways opposites (repetitive and stereotyped vs. impulsive, for example). We hypothesized, therefore, that ethnically and geographically matched autism probands would provide a test of the specificity of rare *DRD4* variants observed in ADHD probands [Grady et al., 2003].

MATERIALS AND METHODS

Autism Sample

Cell lines and DNA were obtained by standard methods [Grady et al., 2003] from 68 probands who met criteria for autism on the autism diagnostic observational schedule-generic (ADOS-G) and the autism diagnostic interview (ADI-R) [Lord et al., 1989, 1994]. Briefly, following informed consent, children were enrolled in this study if they met the following criteria: (1) the product of an uncomplicated pregnancy, with gestational age between 37 and 42 weeks, and a

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normal perinatal course, (2) head circumference ≥2nd centile, with normal hearing acuity, (3) no indication of lateralized deficits on neurological examination, orofacial anomalies, genetic syndromic classification including fragile X, or other serious medical condition, (4) no significant head injury with loss of consciousness of greater than 5 min, and (5) no uncontrolled seizures or requisite multiple anticonvulsants. Children who subsequently met ADOS-G and ADI-R criteria for autistic disorder (AD) were included as AD probands for this study. The geographic/ethnic ancestry of the AD probands was similar to prior samples obtained from Orange County, CA (European, 67.6%; Hispanic, 26.5%; Asian, 3.7%; African, 1.5%; and Native American, 0.7%).

DNA Sequencing/Haplotyping

The *DRD4* exon 3 region of each proband was amplified, sequenced and haplotyped as described previously [Ding et al., 2002; Grady et al., 2003; Wang et al., 2004]. Briefly, the VNTR was PCR amplified with primer sets 5′-CGTACTGTGCGGCCTCAACGA-3′and 5′-GACACAGCGCCTGCGTGATGT-3′ (705 nucleotide product for the 4R-allele), and DNA cycle sequencing was conducted using ABI 3100 and 3700 automated sequencers [Riethman et al., 2001]. For heterozygous individuals, gel purification of each allele (for example, 4R and 7R) was conducted prior to cycle sequencing [Grady et al., 2003]. Analysis of sequence data was accomplished using Phred, Phrap, Polyphred, and Consed [Nickerson et al., 1997]. The collection of SNPs into a relational database was done via an in-house software package we have designated SNPMAN [Grady et al., 2003].

RESULTS AND DISCUSSION

DNA obtained from 68 autism disorder (AD) probands was amplified at the *DRD4* VNTR, sequenced and haplotyped as described previously [Ding et al., 2002; Grady et al., 2003; Wang et al., 2004]. Haplotypes of the 136 alleles obtained from these predominantly European ancestry autism probands are shown in Table I, using our prior proposed nomenclature [Ding et al., 2002; Grady et al., 2003]. In this nomenclature, haplotypes are given a series of numbers corresponding to different 48 bp sequence variants. For example, the most common 4R allele is designated 4R(1-2-3-4).

In these AD probands (Table I), only two alleles, not previously identified in a world-wide sample [Ding et al., 2002], were uncovered. These alleles are indicated in bold (Table I). Both alleles were in individuals of European ancestry. One of these novel alleles is an amino acid changing 4R-7R allele recombinant (7R [1-2-6-1-2-14-4]) and the second (7R [1-2-6-5-2-29-4]) is a silent mutation. As discussed in our prior articles [Ding et al., 2002; Grady et al., 2003], independent samples of this size (N = 136) would be expected to uncover, at most, one or two novel *DRD4* alleles from the hundreds of rare alleles (<0.01 frequency) likely to exist. The remaining 134 alleles derived from AD probands (Table I) were found at similar frequencies in our prior population sample [Ding et al., 2002]. European ancestry control alleles (N = 220), selected from

TABLE I. Haplotypes of *DRD4* Exon3 Alleles

European ancestry (N = 220) [Ding et al., 2002]			Autism (N = 136) (this study)		ADHD (N = 250) [Grady et al., 2003]	
Allele	N	Haplotype	N	Haplotype	N	Haplotype
2R	30	1-4	14	1-4	23	1-4
3R	8	1-7-4	3	1-7-4	8	1-7-4
	4	1-9-4			3	1-9-4
	1	1-2-31			2	**1-2-20**
	1	1-2-21			1	**1-6-4**
4R	90	1-2-3-4	90	1-2-3-4	150	1-2-3-4
	2	1-2-14-4	2	1-8-3-4	2	1-2-14-4
	1	1-8-3-4	1	1-9-3-4	2	**1-2-5-4**
					1	**1-2-6-4**
					1	**1-26-3-4**
5R	1	1-3-2-14-4	1	1-11-2-3-4	0	
	1	1-2-6-23-4				
6R	6	1-2-3-2-3-4	1	1-2-3-2-3-4	1	1-2-3-2-3-4
	1	1-2-6-5-2-20			1	1-2-6-5-2-20
	1	1-2-6-5-2-4			1	**1-2-6-2-5-4**
7R	64	1-2-6-5-2-5-4	21	1-2-6-5-2-5-4	45	1-2-6-5-2-5-4
	2	1-8-25-5-2-5-4	1	1-2-6-5-2-3-4	2	1-2-6-5-2-5-19
	1	1-2-6-5-2-3-4	1	**1-2-6-1-2-14-4**	2	**1-2-6-1-2-3-4**
	1	1-2-6-5-2-13-4	1	**1-2-6-5-2-29-4**	1	**1-2-6-5-2-23-4**
	1	1-2-6-5-2-5-19			1	**1-2-6-5-8-5-4**
	1	1-2-6-2-2-5-4			1	**1-2-14-5-2-5-4**
					1	1-2-3-17-2-5-4
					1	1-8-25-5-2-5-4
					1	1-2-6-5-2-3-4
8R	1	1-2-6-26-5-26-3-35	0		1	**1-2-6-26-5-2-3-4**
	1	1-2-6-26-5-26-3-4				
	1	1-2-6-5-2-2-5-4				
9R	0		0		1	**1-8-25-5-2-5-2-23-4**

N, allele number identified by DNA sequence analysis; haplotype nomenclature is described in Ding et al. [2002], where individual numbers (i.e., 1-2-3-4) refer to distinct *DRD4* exon 3, 48 bp repeat sequence motifs. Alleles in normal font were identified previously in a survey of 600 worldwide alleles [Ding et al., 2002]. Alleles in bold font are unique to this study (center column) or our prior ADHD study [last column; reproduced from Grady et al., 2003]. Genotypes of the autism probands (N = 136 center column) were as expected from this predominantly European (including Hispanic) ancestry population (2R/2R = 1, 2R/4R = 10, 2R/7R = 2, 3R/4R = 3, 4R/4R = 30, 4R/5R = 1, 4R/6R = 1, 4R/7R = 18, 7R/7R = 2). European ancestry alleles (N = 220) selected from the [Ding et al., 2002] study are shown for comparison (first column). In that study, non-4R alleles were oversampled approximately 2-fold in comparison to their population frequency. The alleles observed in ADHD probands from the Grady et al. [2003] study are also shown for comparison (last column).

the 600 alleles in this prior study are shown in Table I for comparison.

Note that these control alleles were not randomly selected, but contain an approximate twofold "over sampling" of non-4R alleles, that is, those disproportionately found in ADHD probands [Grady et al., 2003]. The genotype distribution of *DRD4* variants in European ancestry populations is 2R = 0.07, 3R = 0.03, 4R = 0.73, 5R = 0.01, 6R = 0.02, 7R = 0.12, 8R < 0.01, 9R < 0.001 [2N = 1652; Grady et al., 2003]. The autism haplotype distribution of *DRD4* alleles, then, is that expected for an independent sample representing a largely European ancestry population, where only 1.5% of the observed alleles (2/136) are novel (Table I).

In contrast, our recent ADHD *DRD4* haplotype results [Grady et al., 2003] uncovered a four-fold greater number of novel alleles (15/250 = 6%; reproduced in column three of Table I). Given that these ADHD and AD probands were collected in the same geographic area and are of similar ethnic composition and age [Table I; Grady et al., 2003], we propose that these results provide additional support for the hypothesis that allelic heterogeneity at the *DRD4* VNTR is playing a role in the association of this gene and ADHD. Further, it suggests that other *DRD4* polymorpisms tightly linked to the 7R VNTR [Ding et al., 2002; Wang et al., 2004] are unlikely to be the "cause" of the *DRD4*-ADHD association. It is extremely unlikely that an adjacent "causative" polymorphism would preferentially be associated with numerous rare *DRD4* variants, given the independent origin of these rare variants [Ding et al., 2002: Wang et al., 2004].

These results (Table I) also suggest that variations in the *DRD4* exon 3 polymorphism are unlikely to play a major role in the etiology of autism. However, we cannot rule out an association with other high-heterozygosity polymorphisms present in the *DRD4* gene [Wang et al., 2004] without further studies.

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