

IMMEDIATE COMMUNICATION

Association of the dopamine receptor D4 (DRD4) gene with a refined phenotype of attention deficit hyperactivity disorder (ADHD): a family-based approach

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Keywords: attention deficit hyperactivity disorder; dopamine; DRD4; genetics

Previously in this journal, we reported an association of the dopamine D4 receptor gene (DRD4) and attention deficit hyperactivity disorder (ADHD). In a population-association (case-control) study of 39 children with a refined phenotype of ADHD and 39 ethnically matched controls, we observed an increased percentage of the 7 repeat allele (29% vs 12%) and the 7+ genotype (49% vs 21%) in the ADHD group compared to the control group. In a replication and an extension of our initial study, we recruited another sample of ADHD subjects and found percentages of the 7 repeat allele (28%) and the 7+ genotype (48%) consistent with our previous findings. We used a family-based approach to evaluate a predicted association of DRD4 and ADHD based on a test of allele transmission focused on the 7 repeat allele. We identified 52 families based on the diagnosis of the refined phenotype of ADHD in the proband and the availability of DNA from both biological parents as well as the proband. Haplotype relative risk (HRR) analysis was performed to test our *a priori* hypothesis and produced significant results (chi-square = 4.65, $P < 0.035$). This provides additional evidence that the DRD4 gene is associated with a refined phenotype of ADHD.

In recent investigations of genetic etiologies of ADHD,¹ we have focused on the coding region of the DRD4 gene located on chromosome 11p.² This is a primary candidate gene for association with ADHD for several reasons. First, the most common treatments for ADHD are the stimulant medications (methylphenidate, amphetamine, and pemoline) that act primarily on the dopamine system, implicating DRD4 and other dopamine genes. Second, DRD4 displays a high degree of variability in the third cytoplas-

mic loop of the protein due to a 16-amino acid (48-bp) region that can be repeated two to eleven times.³ Third, *in vitro* studies suggest that the D4 receptor variants display functionally different pharmacological properties.⁴ Fourth, the DRD4 gene^{5,6} has been associated with the personality trait of novelty seeking as defined by Cloninger,⁷ which may be related to the manifestation of symptoms of ADHD. Fifth, the localization of DRD4 mRNA in frontal and prefrontal cortical regions of the brain,⁸ suggest that this gene may be involved in the executive control and regulation of attention.⁹

In our initial evaluation of the DRD4 gene and ADHD,¹ we observed a higher percentage of the 7-repeat DRD4 allele in an ADHD group (29%) compared to the ethnically-matched control group (12%). In addition, we observed a higher percentage of cases with at least one 7-repeat allele (which we called the 7+ genotype) in the ADHD group (49%) than in the control group (21%). We recognized several limitations in our initial study. Although our control group was carefully matched for ethnicity and showed the allele frequency expected for the population studied,^{10,11} the interpretation of our findings from a case-control study was limited by the potential for spurious positive findings due to population stratification.¹² A family-based approach,¹³ in which the non-transmitted parental alleles are used to define a hypothetical and perfectly matched control group for evaluating allele transmission, overcomes this problem.^{14–16} The statistic for this family-based association design is the haplotype relative risk (HRR) contingency coefficient.

In the present study, we used a family-based approach^{12–16} to replicate and extend our initial study in a new sample of families identified by recruitment for a series of clinical trials designed to evaluate stimulant medications used to treat ADHD. The inclusion criteria for subjects to be eligible for these clinical trials (sponsored by ALZA Corporation, Palo Alto, CA, USA and Richwood Pharmaceuticals, Florence, KY, USA), and thus to be probands in our genetic study, included a clinical diagnosis of ADHD by DSM-IV criteria confirmed by a structured interview, current treatment with typical clinical doses of methylphenidate (the most commonly used stimulant medication), and absence of serious comorbid disorders. We identified 119 families in which the proband met these criteria for a refined phenotype of ADHD. These families signed consent forms to participate in a separate study on the genetics of ADHD and to contribute blood samples for DNA extraction.

We were able to obtain blood samples, extract DNA and obtain DRD4 genotypes from 136 parents and from 105 probands. In 75 of these families, we could deduce transmission of the DRD4 alleles from 127 parents to the 75 probands. In 52 of these families, we had complete information on both parents as well as the proband (ie, the DRD4 alleles of trios for the HRR analysis). As shown in Table 1, the percentage of 7 repeat alleles in the probands (29/104 = 28%) was about the same as in our prior sample (23/78 = 29%). Also, the percentage of probands with a 7+ genotype

Table 1 Frequencies and percentages of DRD4 alleles for 104 parents and 52 probands

No. of repeats	2	3	4	5	6	7	Total
Parents							
Frequency	15	4	138	4	0	47	208
Percentage	7.2%	1.9%	66.3%	1.9%	0%	23.4%	100%
Probands							
Frequency	8	1	66	0	0	29	104
Percentage	7.7%	1.8%	63.5%	0%	0%	27.9%	100%
Controls							
Frequency	7	3	72	4	0	18	104
Percentage	6.9%	2.9%	69.2%	3.8%	0%	17.3%	100%

(25/52 = 48%) was about the same as in our prior sample (19/39 = 49%).

We also show in Table 1 the allele frequencies and percentages for alleles in the parents, as well as for the theoretical controls derived by subtraction of the allele frequencies of the probands from the allele frequencies of the parents. In this family-based association study, this 'control' group does not provide an estimate of population allele frequency, but instead provides a control for population stratification that may be present in the sample.

In our family-based association design, the HRR analysis of parent-to-proband allele transmission provides a test for association of an allele (7 repeat) with a disorder (ADHD), and the parental alleles are the focus of analysis.¹²⁻¹⁶ The frequencies and percentages of the observed allele pairs from the 104 parental chromosomes, with transmission (t) and non-transmission (nt) noted, are shown in Table 2.

From the frequencies in Table 2, the HRR statistic was calculated using the method described by Ewen and Spielman.¹² The '4n' transmitted and non-transmitted alleles from the parents of the 'n' trios were used to form Table 3. The HRR contingency statistic [$HRR = 4n(w-y)^2 / ((w+y)(4n-w-y))$] was significant (chi-square = 4.65, d.f. = 1, $P < 0.035$).

Only 50% of our ADHD probands had a 7 repeat allele of the DRD4 gene, so this is not a necessary condition for the diagnosis of ADHD. As discussed in the introduction, we have focused on the DRD4 gene because of the plausible hypothesis that the 7 repeat allele may encode a subsensitive form of the DRD4

Table 3 HRR analysis of the 52 parent-proband trios

	Parental allele type		
	7+	7-	Total
Transmitted	30 (w)	74 (2n - w)	104 (2n)
Non-transmitted	17 (y)	87 (2n-y)	104 (2n)
Total	47 (w+y)	161 (4n-w-y)	208 (4n)

receptor, which may produce underactivity in cortical-basal ganglia dopamine pathways^{9,17,18} and may be associated with personality traits (eg, novelty seeking) that contribute to the manifestation of ADHD.⁵⁻⁷ However, the candidate gene approach we used and our focus on the DRD4 gene would not detect the contributions of other genes. We have taken the first steps to investigate other dopamine genes,¹⁹ including the dopamine transporter gene (DAT1), which Cook *et al*²⁰ and Gill *et al*²¹ found to be associated with ADHD, and the dopamine D2 receptor gene (DRD2), which Comings *et al*²² found to be associated with ADHD and many other psychiatric disorders. In our studies based on the refined phenotype of ADHD described here, we have not found significant association with the DAT1 and DRD2 genes.¹⁹ This may be due to our use of a refined phenotype of ADHD (based on the absence of comorbid conditions and the presence of clinical response to methylphenidate), to our use of an inadequate sample size, or to other factors. In the next step in our research program, we intend to identify affected relatives and use a genome scan design²³ to identify other genes that may be associated and linked to our refined phenotype of ADHD.

We are also aware that ADHD may have non-genetic as well as genetic etiologies. In fact, in adoption²⁴ and family²⁵ studies of ADHD, models have been constructed that indicate a high phenocopy rate and suggest that in clinical samples about half of the cases may be phenocopies and not have a genetic basis for the disorder. It has been suggested that environmentally-altered brain development due to fetal distress, which selectively damages dopamine neurons²⁶ and affects cortical-basal ganglia dopamine pathways,^{27,28} may increase risk for behavioral symptoms of ADHD. We hypothesize that similar biological consequences (eg, underactivity in cortical-basal ganglia neural networks) may occur due to genetic factors (eg, inheritance of a

Table 2 Frequencies and percentages of observed allele pairs in the 104 parents

Alleles	3t4nt	2t4nt	4t2nt	4t3nt	4t4nt	4t5nt	4t7nt	7t4nt	7t2nt	7t5nt	7t7nt
Frequency	1	9	4	3	41	3	13	23	2	1	4
Percentage	1.0%	8.7%	3.8%	2.9%	39.4%	2.9%	12.5%	22.1%	1.9%	1.0%	3.8%

7+ genotype that produces a subsensitive dopamine D4 receptor variant) or to non-genetic factors (eg, damage to striatal dopamine neurons during fetal distress).

We should emphasize that in the present study, we continued to use a narrowly defined phenotype of ADHD. This refined phenotype has been characterized as the overlap of the DSM-IV²⁹ diagnosis of ADHD and the ICD-10³⁰ diagnosis of Hyperkinetic Disorder (HKD), and the term ADHD/HKD has been suggested to identify the subset of ADHD cases who also meet HKD criteria.³¹ The ADHD/HKD phenotype is based on diversity of symptoms (cases with fewer than six inattention symptoms, three hyperactivity symptoms, and one impulsivity symptom are excluded, so many of the cases with the Primarily Inattentive or Primarily Hyperactive/Impulsive subtypes specified in DSM-IV do not meet these refined diagnostic criteria) and an absence of comorbid disorders (ADHD cases with concurrent diagnosis of anxiety, depression, pervasive developmental or Tourette's disorder are excluded, so many of the comorbid subgroups allowed in DSM-IV do not meet these refined diagnostic criteria).

Also, we should emphasize that we continued to limit our studies to children being treated with stimulant medication and manifesting a beneficial clinical response. As recently confirmed by anatomical brain imaging,³² the requirement of a beneficial response to stimulant medication may identify a group of ADHD cases with reduced size of the executive control neural network defined by Posner and Raichle.³³ This network is localized to anterior cingulate and basal ganglia brain regions^{9,17,31-33} where D4 receptors may be concentrated.⁸

In summary, the data from this study provide additional evidence that the DRD4 gene is associated with a refined phenotype of ADHD. Despite the improvement in methodology provided by the family-based association design, our findings still need to be replicated in independent and larger samples. We recommend that the refined phenotype of ADHD, which we used in our present study and our prior study,¹ be considered in attempts to replicate our finding of an association of the DRD4 gene with ADHD.

Methods

Clinical

ADHD probands were recruited for participation in a clinical trial being conducted at the UC Irvine Child Development Center and sponsored by ALZA Corporation or Richwood Pharmaceuticals. The criteria for eligibility included a confirmed diagnosis of ADHD and absence of comorbid diagnoses of anxiety, mood disorder, pervasive developmental disorder, conduct disorder, Tourette's disorder, or other conditions requiring separate, non-stimulant pharmacological treatment or special placement in school due to extremely aggressive behavior. These diagnostic criteria were documented by structured interview with the Diagnostic Interview Schedule for Children (DISC).³⁴ The age at assessment was between 7 and 12

years. At least a 3-month history was required of treatment with and clinical response to approved doses of methylphenidate (not more than 20 mg per administration and not more than 60 mg per day). An IQ score of at least 85 was required and was verified by the use of the Wechsler Intelligence Scale for Children, Version III (WISC-III). Severity of symptoms endorsed on the DISC interview was documented by ratings of the DSM-IV symptoms on the SNAP rating scale³⁵ completed by teachers as well as parents of the probands.

Between May 1996 and August 1997, 813 families were screened and 191 met the entry criteria for participation in our clinical trials program. By September 1997, 119 of these families participated in assessment phases of a clinical trial and agreed to participate in this separate genetic study. We were able to obtain a blood sample and extract DNA from 105 probands and 136 parents. In this sample, the average number of symptoms endorsed on the DISC was 7.8 from the Inattention domain and 7.5 from the Hyperactive/Impulsive domain. The average initial dose of methylphenidate was 14.5 mg, the average number of administrations per day was 2.3, and the average daily dose was 28.9 mg. The percentage of probands with ADHD combined type was 82%, and 47% also met DSM-IV criteria for Oppositional Defiant Disorder. We excluded cases with a primary diagnosis of comorbid Tourette's Disorder, Conduct Disorder, Anxiety Disorder, Mood Disorder, or Pervasive Developmental Disorder.

Genetic

Blood samples were obtained when families were being seen at the UCI Child Development Center. These samples were sent to the Clarke Institute of Psychiatry, Toronto, Canada for DNA extraction. Within 3 days, genomic DNA was extracted from whole blood by salting out. The amplification of the 48-base pair repeat region of DRD4 was performed as described by Lichten *et al.*³⁶ PCR products were separated in 3.5% agarose gels prepared with ethidium bromide and electrophoresed in 1× TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA Na₂).

Statistical

Genotypes were obtained from available DNA of probands and parents, and transmission of alleles from parent to proband was determined for 75 probands. In 52 of these families, genotypes were obtained on trios of the proband and both parents. The HRR test was used to calculate a within-family contingency statistic based on the alleles of the 'n' (52) trios. The '2n' transmitted alleles, consisting of 'w' 7+ alleles and '2n - 2' 7-alleles, and the '2n' non-transmitted alleles, consisting of 'y' 7+ alleles and '2n - (2n - y)' 7-alleles, were used to calculate the within-family contingency coefficient by the following formula: $HRR = 4n(w-y)^2 / [(w+y)(4n-w-y)]$.

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Received 15 October 1997; revised 30 October 1997; accepted 31 October 1997