

Polymorphisms in the dopamine D4 receptor gene and attention-deficit hyperactivity disorder

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There is considerable evidence to support a role of dopamine-related genes in the molecular aetiology of attention-deficit hyperactivity disorder (ADHD). A 48 bp repeat in exon three of the dopamine D4 receptor gene has been widely studied in clinical ADHD samples, and a meta-analysis of published studies suggests it is associated with ADHD. A number of other polymorphisms across this gene have been characterised but not so thoroughly investigated in relation to ADHD. In this study we have genotyped five polymorphisms (a 120 bp promoter-region duplication, the

–616 C/G substitution, the –521 C/T substitution, a poly-G repeat in intron I, and the 48 bp exon 3 repeat) across the gene in a large clinical sample ($n = 188$) and their families. We found that none of the markers is individually associated with ADHD, although there is evidence to suggest that a haplotype of markers in the 5' promoter region of the gene (allele 2 of the 120 bp duplication, the C allele of the –616 substitution, and the C allele of the –521 substitution) may confer susceptibility. *NeuroReport* 14:1463–1466 © 2003 Lippincott Williams & Wilkins.

Key words: Association study; Attention-deficit hyperactivity disorder (ADHD); DRD4; Genetics; Haplotypes; Linkage disequilibrium; Promoter

INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is one of the most prevalent, stable, and heritable forms of childhood psychopathology. It is characterised by hyperactivity, impaired attention, and impulsivity and is known to be a significant risk factor for psychopathology later in adult life [1]. While the exact etiology of ADHD is not known, it has been shown to have a high genetic component [2]. Evidence from neuroimaging, animal models, and the pharmacological effects of stimulant medication suggests that dysregulation in catecholamine neurotransmission, in particular of dopamine, is strongly implicated in ADHD. The majority of molecular genetic studies have thus focused on genes involved in dopamine neurotransmission, with replicated findings for the dopamine transporter gene (DAT1), the dopamine D4 receptor gene (DRD4) and the dopamine D5 receptor gene (DRD5) [3]. Perhaps the most consistent findings in ADHD genetics have been with the DRD4 gene, which is located at 11p15.5. DRD4 was first cloned in 1991 and found to have high homology with two other dopamine receptor genes, DRD2 and DRD3 [4]. Its high affinity for the antipsychotic drug clozapine means it has been one of the most widely studied genes in neuropsychiatric genetics. The most intensely studied DRD4 polymorphism is a 48 bp variable number of tandem repeats (VNTR) polymorphism in the third exon identified by Van Tol *et al.* [5]. This encodes a portion of the third intracellular loop region of the transcribed protein that spans the nerve cell membrane and

mediates interaction with secondary signalling proteins. The number of repeats ranges from 2 to 11, and although the functional significance of this polymorphism is yet to be ascertained, evidence suggests that different D4 receptor variants may display different pharmacological properties [6]. Numerous published clinical ADHD studies have shown association between the 7-repeat allele of DRD4 and ADHD. A recent meta-analysis of the association between DRD4 and ADHD using both published and unpublished data suggest there is a real, but small, effect. Faraone *et al.* calculated an odds ratio (OR) of 1.9 (95% CI: 1.4–2.2, $p = 0.00000008$) from seven case-control studies and 1.4 (95% CI: 1.1–1.6, $p = 0.02$) from 14 family-based studies [7]. It is important to note, however, that there have been several non-replications of this association ([8] and references therein).

It is thus possible that in some, but not all populations, the 7-repeat is in linkage disequilibrium (LD) with another, functional variant, such as the –521 (C/T) DRD4 promoter polymorphism, reported to reduce transcriptional activity by ~40% and associated with the personality traits of novelty seeking and impulsivity [9]. Evidence to support the theory that the effect of DRD4 on hyperactivity is mediated not by the exon 3 VNTR but by a functional variant in the promoter region of the gene comes from a recent study by McCracken *et al.* [10]. They found a highly significant association with the presence of a 120 bp duplication in the promoter of DRD4, but no significant association with the

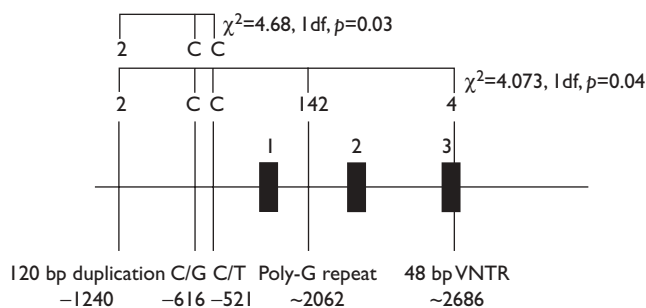


Fig. 1. Relative positions of the DRD4 markers genotyped in this study, and the putatively associated promoter haplotype showing biased transmission to ADHD probands.

exon 3 VNTR. However, a subsequent study failed to replicate this finding [11]. A number of other polymorphisms have also been detected across the DRD4 gene, including a poly-G repeat in intron 1 [12].

In this study, we have genotyped a large clinical sample of ADHD probands and their families for five markers in DRD4. The markers chosen were the 120 bp promoter duplication, two SNPs in the promoter (–616 C/G and –512 C/T), the poly-G repeat in intron 1, and the VNTR in exon 3. The relative positions of these polymorphisms can be seen in Fig. 1. We have previously genotyped the exon 3 VNTR in a subset of our clinical sample and found no evidence from a family-based study design to implicate this polymorphism in ADHD, although we did find evidence for an association using a case-control design [13]. The aim of this study was to elucidate more precisely the region of the gene, if any, associated with ADHD by examining for biased transmission of specific alleles and multi-marker haplotypes to affected probands.

MATERIALS AND METHODS

Sample: In total, DNA from 188 probands and their families were used for this study. DNA was available from both parents for 121 of the families, and from only the mother in 64 families. At least 1 sibling who was also genotyped was available for 113 of the affected families. Cases were referred for assessment if they were thought by experienced clinicians to have a diagnosis of the combined subtype of ADHD under standard diagnostic criteria, with no significant Axis I co-morbidity apart from oppositional defiant disorder and conduct disorder. Parents of referred cases were interviewed with a modified version of the Child Assessment Parent Interview. Information on ADHD symptoms at school were obtained using the Conners questionnaire. All the subjects used in this study were free of neurological disease and damage, and did not have any congenital disorders known to cause hyperactivity. Out of 188 cases included in this study, 176 had the combined subtype, eight had the hyperactive/impulsive subtype and four the inattentive subtype. DNA was obtained using buccal swabs as described in Freeman *et al.* [14].

Genotyping: The exon 3 VNTR was amplified as described by Mill *et al.* [13] and PCR products were run out on a 2%

agarose gel stained with ethidium bromide and analysed under u.v. light. The 120 bp promoter duplication was genotyped as described by Seaman *et al.* [15] and PCR products were run out on a 2% agarose gel stained with ethidium bromide and analysed under u.v. light. The two promoter region SNPs were contained in a region amplified by the primers 5'-TCAACTGTGCAACGGGTG-3' and 5'-GAGAAACCGACAAGGATGGA-3' as described by Barr *et al.* [11]. The –521 C/T SNP was assayed by digesting the PCR product with 5 units *FspI* and running out the fragments on a 2% agarose gel stained with ethidium bromide. Allele C resulted in no cutting of the product whereas allele T resulted in two fragments of 228 and 152 bp. The –616 C/G SNP was assayed by digesting the PCR product with 5 units *BspI* and running out the fragments on a 2% agarose gel stained with ethidium bromide. Allele G resulted in no cutting of the product whereas allele C resulted in two fragments of 50 and 330 bp. The intron one poly-G repeat was genotyped as described by Petronis *et al.* [12]. The forward primer was labelled with the fluorophore FAM at its 5' end and fluorescently tagged products were separated on an ABI 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) and analysed using Genotyper (PE Applied Biosystems, Foster City, CA, USA) software.

Analysis: Family genotype data was analyzed using David Clayton's program TRANSMIT (version 5.4), which is available for download from the web at <http://www.gene.cimr.cam.ac.uk/clayton/software/>. TRANSMIT tests for association between genetic markers and disease by examining the transmission of markers from parents to affected offspring. The main features of TRANSMIT, which differ from other similar programs, are that it can deal with transmission of multi-locus haplotypes, even if phase is unknown, and that parental genotypes may be unknown. The tests are based on a score vector, which is averaged over all possible configurations of parental haplotypes and transmissions consistent with the observed data. Data from unaffected siblings (or siblings whose disease status is unknown) may be used to narrow down the range of possible parental genotypes that need to be considered, thus maximising the power of our sample to detect an association with any of the DRD4 markers tested. The program Simwalk2 (version 2.83) written by Eric Sobel and available online at <http://watson.hgen.pitt.edu/docs/simwalk2.html> was used to infer haplotype structure and compute LD relationships between each of the markers. LD relationships were also assessed in the parental samples using the program 2LD, written by Jing Hua Zhao, and available online at <http://www.iop.kcl.ac.uk/iop/Departments/PsychMed/GEpiBSt/software.html>.

RESULTS

Using the parental chromosomes, allele frequencies for each of the five markers were comparable to those reported elsewhere in the literature. Allele 2 of the DRD4 120 bp repeat was more common than allele 1 (78% vs 22%). The C allele of the –616 C/G SNP was more common than the G allele (65% vs 35%) and the T allele of the –521 C/T SNP slightly more frequent than the C allele (53% vs 47%). Eight

Table 1. Linkage disequilibrium between the five DRD4 markers.

Marker 1	Marker 2	D'	χ^2	p
120 repeat	-616 C/G	0.142	4.559	0.0328
120 repeat	-521 C/T	0.069	0.907	0.3409
120 repeat	Intron 1 poly-G	0.284	68.056	0.000
120 repeat	Exon 3 VNTR	0.243	38.711	0.000
-616 C/G	-521 C/T	0.084	3.645	0.0562
-616 C/G	Intron 1 poly-G	0.101	8.091	0.0442
-616 C/G	Exon 3 VNTR	0.060	3.372	0.3377
-521 C/T	Intron 1 poly-G	0.069	3.957	0.2662
-521 C/T	Exon 3 VNTR	0.149	19.219	0.002
Intron 1 poly-G	Exon 3 VNTR	0.560	350.787	0.000

Table 2. TRANSMIT output for common (frequency > 5%) DRD4 five-marker haplotypes.

Haplotype	Observed	Expected	Untransmitted	χ^2
2-C-T-142-4	47.19	48.19	49.65	0.09
2-G-T-142-4	34.25	36.23	38.21	0.29
2-C-C-142-4	31.31	25.19	19.06	4.07
2-G-C-142-4	20.80	23.87	26.95	1.04
2-C-T-140-7	18.24	19.77	21.29	0.36

alleles of the intron poly-G repeat were detected, although by far the most common was the 142 bp allele with a frequency of 61%. Finally, two to eight repeats of the exon 3 VNTR were observed with the frequencies of the two most common alleles, 4 and 7 repeats, being 62% and 20%, respectively. LD relationships between the five markers are outlined in Table 1. In agreement with previous studies [11] we found relatively low levels of LD (as measured by D') between the promoter region markers, with the highest degree of LD being seen between the intron 1 poly-G repeat and the exon 3 VNTR ($D' = 0.56$, $\chi^2 = 0.19$).

Using TRANSMIT we found no evidence of biased transmission to ADHD probands of any of the alleles of each of the five individual markers, suggesting that none of the five markers was individually associated with ADHD in this sample. We then decided to investigate haplotypes of the DRD4 markers. We limited haplotype-based tests to haplotypes present with a frequency greater than 5%, as in general less frequent haplotypes were too uncommon to provide sufficient information. Looking at all the markers together, we found five common haplotypes (> 5% frequency) in this sample (Table 2). One haplotype (2-C-C-142-4) showed marginal evidence for biased transmission to ADHD probands ($\chi^2 = 4.073$, 1 df, $p = 0.04$). We next decided to focus in on the three markers in the promoter region of the gene. Again we found marginal evidence to suggest that one haplotype (2-C-C), which forms part of the associated 5-marker haplotype, was over-transmitted to affected ADHD probands ($\chi^2 = 4.68$, 1 df, $p = 0.03$). These data are summarised in Fig. 1.

DISCUSSION

None of the five polymorphisms genotyped across the DRD4 gene gave evidence for significant association with ADHD when considered individually. We therefore found no evidence to support the meta-analysis of the exon 3

VNTR [7] or the findings of McCracken *et al.*, who found an association with the 120 bp duplication [10]. However, a haplotype of the three promoter polymorphisms (allele 2 of the 120 bp repeat, allele C of the -616 C/G SNP and allele C of the -521 C/T SNP) appears to show some evidence of association with ADHD in this sample when considered on its own or in conjunction with the most common alleles of the other two polymorphisms to form a five-marker haplotype. While it thus appears that none of these promoter variants is increasing risk to ADHD on their own, it is possible that chromosomes carrying this specific associated haplotype may also carry a functional variant at another locus that is increasing risk to ADHD. The fact that the association for this three-marker haplotype is as strong on its own as when combined with the other two markers genotyped in this study suggests that the putative functional variant causing this effect is likely to be located in the 5' region of the DRD4 gene. These findings should, however, be treated with caution. By definition the analysis of haplotypes involves a degree of multiple testing, and these data need to be replicated before any real conclusions can be made.

The DRD4 gene has been fairly consistently associated with ADHD and other related personality traits and behaviours. Most studies have focused singly on the exon 3 VNTR, and a meta-analysis of this polymorphism demonstrates a small, but significant, association [7]. A number of studies, however, concur with us in failing to replicate this finding [8]. A number of factors could affect our ability to detect an association between this, and other previously associated individual DRD4 markers, and ADHD. For example, it is likely that our sample does not necessarily have the power to detect what have been reported to be small effect sizes. Faraone *et al.* have calculated that the odds ratio for the exon 3 VNTR is only 1.4 from a transmission-based analysis of 1665 trios [7]. We have estimated that a sample size of ~500 complete trios

would be needed to detect this size effect, assuming 80% power and an alpha level of 0.05. It is often stated that this polymorphism exerts a real functional effect. Asghari *et al.* report that different repeat lengths confer different pharmacological properties, with the 7 repeat acting to blunt the cellular response to dopamine [6]. Subsequent studies, however, have not replicated these findings, and there is no conclusive evidence to suggest that this polymorphism exerts any functional effect at all [16–18]. Another possible cause of the failure to replicate the findings of the DRD4 exon 3 VNTR have been raised by the findings of Grady *et al.* (in press). They have resequenced the exon 3 VNTR in 132 ADHD probands, and found a high degree of novel variation. Such allelic heterogeneity within the VNTR itself could explain the variable findings from different groups. It is possible that the relative risk of this locus has been greatly underestimated by simply focussing on repeat length rather than DNA sequence.

McCracken *et al.* were the first group to find an association of the 120 bp duplication in DRD4 with ADHD [10]. Interestingly, when they also looked at the exon 3 VNTR in conjunction with the 120 bp repeat, they found that this association was no stronger, suggesting that the duplication itself, or a sequence closely linked, may be the real associated variant. A recent study has demonstrated a strong functional effect of this repeat on levels of transcription in a transient *in vitro* expression assay [19]. Another of the polymorphisms contained within our putatively associated haplotype has also been shown to have functional effects [9]. The T allele of the –521 C/T SNP reduces transcriptional activity by ~40%. Interestingly, this study also reports an association between the C allele of this polymorphism and the personality traits of novelty seeking and impulsivity, which are correlated strongly with the symptoms of ADHD.

It should be noted, however, that Barr *et al.* found no evidence to suggest the 5' promoter region of DRD4 is associated with ADHD, although they did find an association with the 7 repeat allele of the VNTR [11]. To our knowledge, there are no published studies that report significant findings for both the exon 3 VNTR and promoter region in the same sample group. Given the overall consistent findings from other studies into the VNTR polymorphism it is possible that two functional sites exist across the DRD4 gene. Alternatively, it is possible that there is a functional variant located between the two associated regions of DRD4. Different LD relationships in different

population groups may explain why this is being picked up by 5' markers in some studies and the genotype at the exon 3 VNTR in other studies. Future work should thus concentrate on resequencing the DRD4 gene to discover novel functional variants that can explain the consistent associations of this gene with ADHD and related traits.

CONCLUSION

None of the five DRD4 polymorphisms typed in this large clinical ADHD sample was individually associated with the disorder, although a haplotype comprising of alleles of three promoter-region polymorphisms was found to show significantly higher than expected biased transmission to affected probands. These results suggest that while none of the polymorphisms investigated in this study is exerting an independent risk to developing ADHD, there may be an as yet uncharacterized functional variant contained in the 5' region of DRD4. The data underscore the need for a more thorough characterization of this gene and the elucidation of the functional consequences of candidate polymorphic sites.

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