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A cluster of single nucleotide polymorphisms in the 5′-leader of the human dopamine D3 receptor gene (*DRD3*) and its relationship to schizophrenia

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Abstract

The association between schizophrenia and the Ser9Gly variant of the dopamine D3 receptor gene (*DRD3)* has been the subject of numerous studies. Under meta-analysis this site, or one or more in linkage disequilibrium with it, appears to contribute a small increase to the relative risk of schizophrenia. In this study, 768 bp of the 5′-leader region of *DRD3* mRNA was screened for polymorphisms to assess their contribution to the association of *DRD3* with schizophrenia. A cluster of three single nucleotide polymorphisms (SNPs) was identified in tight linkage disequilibrium with each other and with the Ser9Gly polymorphism. One of the 5′-leader SNPs encodes a Lys9Glu variant within a 36 amino acid residue stretch of an upstream open reading frame (uORF). Two common haplotypes are found in the population examined; one is linked to the Ser9 coding variant and the other to the Gly9 variant. A panel of 73 schizophrenic patients and 56 matched controls recruited from the East Anglia region of the United Kingdom was screened for disease association at these sites. Since the 5′-leader and coding sites are in tight disequilibrium, the combined genotype of all 4 sites was scored for each patient. A significant association was seen between disease and the frequency distribution of these genotypes ($\chi^2 = 13.19$, d.f. = 3, $P = 0.0042$; Cochran method for sparse cells applied). A 20% excess of one of the heterozygous genotypes, in which the sequences differ at three of the four SNPs, including Ser9/Gly9 in the receptor and Lys9/Glu9 in the uORF, was found in the patient group. An absence of association of disease with the Ser9Gly polymorphism had previously been reported for this panel. This suggests that these SNPs and the corresponding coding changes may exert a combined or synergistic effect on susceptibility to schizophrenia. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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The dopamine hypothesis of schizophrenia has been the subject of numerous studies. In particular, the suggestion that the dopamine D3 receptor gene (*DRD3)* is a candidate for increased susceptibility to the disease comes from the high affinity of D3 receptors for neuroleptic drugs [12,13]. Higher levels of *DRD3* binding have been found in the mesolimbic system of schizophrenics [8]. A common variant of a single nucleotide polymorphism (SNP) of A/G at position 25 of the *DRD3* coding sequence has been identified [7]. The A substitution (allele 1) encodes a serine at residue 9 whereas the G substitution (allele 2) generates an *Msc*I (or *Bal*I) restriction enzyme site and encodes glycine at residue 9. An excess frequency of homozygotes for both alleles was originally reported in schizophrenic patients [2]. This finding has been followed up by numerous replication studies, some of which have confirmed this conclusion. Others however have not supported it, including our study [3] which employed the same panel of cases and controls that has been used in this study. A meta-analysis [16] in which over 30 of these case-control studies have been examined, concluded that a relationship between schizophrenia and homozygosity for the Ser9Gly polymorphism is present. An *Msp*I polymorphism located 40 kb downstream of the

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*Msc*I site has also been identified, but was not associated with the *Msc*I site or with disease [4].

In all the above studies, it is the frequency of the two variant types of receptor gene that have been assessed in cases and controls, whereas the important factor may be the level of receptor protein production. This is determined by upstream regulatory elements, both in the promoter and in the 5′-leader. To address this problem, we have sequenced 768 bp of the 5′-upstream region of human *DRD3*. Reverse transcription PCR (RT PCR) of this upstream region, using polyA$^+$ mRNA from human adult brain, demonstrated that the 768 bp stretch is situated in the 5′-leader region of the mRNA. This region was then screened for polymorphisms using both single stranded conformation polymorphism (SSCP) and sequencing analysis. Three novel SNPs were identified and their frequency, and that of the MscI polymorphism, were scored in unrelated schizophrenic patients and normal subjects.

As previously reported [3], a panel of unrelated patients of European descent with chronic schizophrenia and age, sex, and ethnically matched controls was recruited from East Anglia, United Kingdom. Informed consent was obtained, subjects were interviewed using the Present State Examination [17] and diagnoses were made using a structured interview with DSM-IIIR criteria to assess recent or chronic psychopathology. Data were also gathered on personal and family psychiatric and medical history; 22% had a first- or second-degree relative with schizophrenia or schizoaffective disorder. Controls were given a semi-structured interview and subjects with any history of major illness, either in themselves or in a first degree relative, were excluded. Symptoms were rated on an ordinal scale for response to clozapine. DNA was extracted from blood using the Nucleon II kit (Scotlab Inc., Shelton, CT).

Seven high-density gridded membranes of a human PAC library (RPC11) from the UK HGMP resource centre were screened with a 219 bp fragment generated by PCR from the 5′ end of the coding region of the *DRD3* gene, using primer pair exon1F and exon1R (Fig. 1). A positive signal was obtained for clone 221/M4. The 5′-flanking region of *DRD3* was then amplified from this clone using the method of single-primer walking PCR [11], initially with primer exon1R to extend upstream. The amplification products were cloned into pTAg (R&D Systems) and sequenced using exon1R as a sequencing primer and ABI PRISM™ dye terminator cycle sequencing. Sequencing was then extended to the 768 bp stretch, as well as to 174 bp of the 5′-coding sequence, using primers designed to the new sequence. All sequencing was carried out on an ABI Model 373a sequencer. The extent of the 5′-leader upstream of the translation start site was examined by generating *DRD3* cDNA from adult human brain polyA+ mRNA (Clontech™) with a Promega™ reverse transcription-polymerase chain reaction (RT PCR) kit and primers 1F and exon1R (Fig. 1). An isotopic PCR-based SSCP method [5] was used to screen from −768 to −150 bp of the 5′-flanking

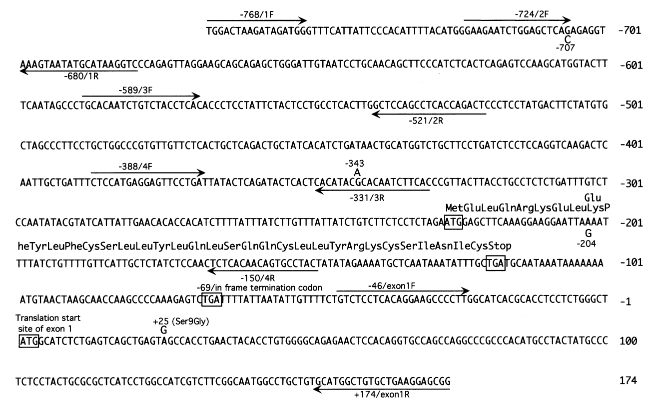

Fig. 1. Sequence of the 5′-leader of *DRD3* mRNA together with 174 bp of the coding sequence. The arrows above and below the sequence indicate the forward (F) and reverse (R) primers used. The position from the translation start site of the 5′-end of each primer is given with the PCR fragment number. The base substitution for each SNP is shown, including the +25 change in the *DRD3* coding sequence that gives rise to Ser9Gly. The 36 amino acids encoded by the uORF are shown with the coding change Lys9Glu.

region of *DRD3* in DNA samples obtained from patients and controls. Overlapping PCR products were generated from four different PCR reactions. The primer pairs used are shown in Fig. 1. The nature of the base changes responsible for the band shifts was determined by sequencing. For each different fragment detected, the sequence of the corresponding PCR product was confirmed with a minimum of 20 individuals comprising at least 10 patients and 10 controls. Statistical comparisons of the patients and controls in $2 \times m$ contingency tables as well as the estimates of relative risk were carried out using standard methods [10]. The SPSS® program was used for the Mann–Whitney–Wilcoxon Rank Sum test for association of clozapine response ratings with genotype.

The sequence to −768 was found to correspond to mRNA. Unfortunately, all attempts to identify precisely the transcription start site with 5′-RACE were unsuccessful. The translation start site in exon 1 was previously deduced from an open reading frame in the sequence of a cDNA clone situated 69 bp downstream of an in-frame termination codon [13]. Our extended sequence (Fig. 1) contains an upstream open reading frame (uORF) between −228 and −120 that would encode a putative 36 residue peptide; the presence of Kozak sequences [6] at the putative start site strengthens the case for the translation of this peptide. No other ORFs were found in this region of the mRNA. SSCP analysis of PCR products 2 and 3 each gave identical banding patterns across all individuals screened, indicating that no polymorphic sites are present. In contrast, fragments 1 and 4 showed a range of banding patterns. PCR product 1 had a single polymorphic site at −707 (C/G), whereas PCR product 4 had two polymorphic sites at −343 (A/G) and −204 (A/G). Assuming random mating, the observed genotypic frequencies for each SNP are not significantly different

from the expected frequencies, suggesting that each is in Hardy–Weinberg equilibrium. The Ser9Gly polymorphism in this panel of patients and controls had previously been found to be in Hardy–Weinberg equilibrium [3].

The identities of all three upstream polymorphic sites were scored for the 73 patient and 56 control DNA samples. The remaining 11 patient and 21 control samples [3] proved refractory to PCR amplification for at least one of the fragments. The level of linkage disequilibium between all four SNP sites, including the Ser9Gly coding A/G variant at +25, was analyzed by the EH program [15], which indicated that the four SNPs are in tight linkage disequilibrium ($P < 10^{-27}$), as expected for sites that lie within an 732 bp stretch of DNA. The combined genotype of all four sites was therefore scored for each patient; the corresponding frequencies of each of the 19 combined genotypes found in the panel are shown in Table 1. Inspection of the mean genotype frequencies of the pooled cases and controls, reveals that two of the genotypes are much more common than the 17 remaining genotypes. Since one of the common genotypes is homozygous at all three upstream sites and for Ser9, this identifies one common haplotype as GGAA. Given the presence of linkage disequilibrium, the other common Gly9 coding haplotype can be identified by subtraction as CGGG. These haplotypes differ therefore at two sites (−707 and −204): one is linked to the Ser9 coding variant and the other to the Gly9 variant of the D3 receptor. The A-204G SNP encodes a non-conservative Lys9Glu substitution in the putative 36 residue peptide encoded by the uORF.

The frequency distribution of the 19 combined genotypes in the patient group compared to the control group was tested by using a 2 × m χ^2 test, for which all cells with expected values <5 were collapsed [11]. A significant difference between cases and controls ($\chi^2 = 13.19$, d.f. = 3, $P = 0.0042$) suggests that there may be an association between these four SNPs and schizophrenia. The relative risk of the two common genotypes was calculated using the odds ratio. The relative risk for individuals that are homozygous for the GGAA haplotype is 1.15 (C.I. 95% = 0.54–2.5), which suggests that this genotype does not affect the susceptibility to schizophrenia. The relative risk of the C/G G/G G/A A/G genotype, which corresponds to heterozygotes for the two most common haplotypes, is 3.13 (C.I. 95% = 1.28–7.6). From inspection, this class is 20% overrepresented in patients. Since these two genotypes were selected post-hoc for the estimation of relative risk, the hypothesis that the heterozygosity for these two haplotypes may increase the risk to schizophrenia remains to be tested in a separate sample. This avoids applying an overly conservative multiple test correction for the 19 genotypes found. The presence of multiple haplotypes in the population examined indicates that these SNPs are sufficiently ancient for recombination to have taken place between sites that are less than 150 bp apart.

The association of clozapine response with the common C/G G/G A/G A/G genotype was assessed by comparing the response ratings of patients on clozapine ($n = 49$) with this genotype versus all other genotypes. No significant association was found ($P = 0.24$); this genotype is not associated therefore with clozapine response in this sample. We have formerly found that patients in this panel either taking or not taking clozapine did not differ significantly in the allele or genotype frequencies of the Ser9Gly variant [3].

Table 1
Frequency distribution of genotypes[a]

5′-Leader SNPs			Coding SNP +25					
SNP	SNP	SNP	Ser9/Ser9 – A/A		Ser9/Gly9 – A/G		Gly9/Gly9 – G/G	
−707	−343	−204	Patients	Controls	Patients	Controls	Patients	Controls
C/G	G/G	A/G	2 (2.7)	1 (1.8)	25** (34.0)	8** (14.0)	0	0
G/G	G/G	A/G	1 (1.37)	4 (7.0)	9 (12.3)	4 (7.0)	0	1 (1.8)
C/C	G/G	A/G	0	1 (1.8)	0	0	0	0
C/G	G/G	A/A	3 (4.0)	3 (5.3)	1 (1.37)	2 (3.6)	0	1 (1.8)
G/G	G/G	A/A	23* (32.0)	16* (29.0)	1 (1.37)	2 (3.6)	0	1 (1.8)
C/G	A/G	G/G	0	0	0	1 (1.8)	0	0
G/G	A/G	G/G	0	0	4 (5.5)	3 (5.4)	0	0
C/G	G/G	G/G	0	0	0	0	2 (2.7)	1 (1.8)
G/G	G/G	G/G	0	0	0	2 (3.6)	1 (1.37)	3 (5.3)
C/C	G/G	G/G	0	0	0	1 (1.8)	1 (1.37)	1 (1.8)
		Totals	29 (40.07)	25 (44.9)	40 (54.54)	23 (40.8)	4 (5.44)	8 (14.3)

[a] Frequency of 5′-leader and coding SNPs in the *DRD3* gene in patients and controls. The number and % frequency in parentheses of each genotype is shown. The Ser9Gly genotypes were determined by Gaitonde et al. (1996) [3]. χ^2 test between 73 patients and 56 controls for all genotypes in the table: $P = 0.004$. Relative risk was estimated by the odds ratio (OR) and 95% confidence interval (CI) for the two most common genotypes, which are indicated by asterisks: *OR = 1.15 (CI 95%, 0.54–2.5) **OR = 3.13 (CI 95% = 1.28–7.6).

The absence of an association between schizophrenia and the Ser9Gly polymorphism in this panel of patients and controls has been reported previously [3], and this subset similarly showed no association with this polymorphism ($P = 0.13$). However, when the SNPs in the coding and 5′-leader are considered together, a significant association with disease is seen. A similar combined 'super-allele' was found by examining the effect of SNPs in the alcohol dehydrogenase (*Adh*) gene on the level of its expression in *Drosophila melanogaster* [14]. This quantitative trait was modulated by the combination of the individual effects of SNPs located in the coding sequence, introns and 3′-untranslated region of *Adh*, that are non-randomly associated. This suggests that when the relationship between polymorphic sites and a complex disease is being assessed for association studies, examination of all of the closely-linked polymorphic sites in combination can be more revealing than when individual sites are considered in isolation. Meta-analysis of the large number of Ser9Gly studies has shown that schizophrenia is associated with homozygosity at this site [16]. However, it is unknown at present whether the Gly9Ser substitution alters the pharmacological properties of the DRD3 receptor. The association of schizophrenia with heterozygosity in this study is dependent on the inclusion of the three 5′-leader sites. This implies that disease susceptibility could be related to different levels of expression of the two protein variants. The 5′-leader may modulate translational activity via the translational control elements as found in the 5′-leader of human GLUT-1 mRNA [1]. However, it remains to be established whether the two common 5′-leader haplotypes of *DRD3* alter the level of protein synthesis. The uORF in this region of the mRNA has similarities to that present in the 5′ leader of the β2-adrenergic receptor mRNA [9]. A peptide rich in basic residues is encoded by this uORF and has been shown to down-regulate expression. Mutations that replace basic for neutral residues in this peptide increased translation by 50%. The presence and role of such a peptide in *DRD3* expression should therefore be investigated. In this regard, it is interesting to note that the uORF adjacent to *DRD3* encodes three basic residues in the first nine N-terminal residues, and that the SNP at −204 would generate a Lys9Glu substitution in this putative peptide.

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