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DRD4 exon III VNTR polymorphism—susceptibility factor for heroin dependence? Results of a case-control and a family-based association approach

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Dopaminergic abnormalities are implicated in the pathogenesis of substance abuse.[1] Recently, two reports have been published suggesting an association between opioid dependence and presence of long alleles of the dopamine D_4 receptor (DRD4) gene exon III VNTR.[2,3] We have attempted to replicate this finding using a two-tiered strategy employing independent case-control and family-based association samples. Our study was possibly the largest candidate gene association study to date on opioid dependence in a sample of 815 subjects, 396 of whom were patients. We found long alleles of the DRD4 exon III VNTR in similar frequency among 285 heroin addicts and 197 controls. Furthermore, no preferential transmission of long alleles to affected offspring was observed in a sample of 111 patients and their parents. Our results, therefore, do not support the hypothesis that alleles of the DRD4 exon III VNTR are susceptibility factors for opioid dependence in man. *Molecular Psychiatry* (2000) **5,** 101–104.

Although social and cultural influences are clearly important, family, twin and adoption studies indicate that genes contribute significantly to substance abuse,[4–6] including addiction to alcohol, cocaine and opioids. In search for genetic factors of opioid dependence, two recent case-control association studies have implicated the DRD4 exon III VNTR in the development of disease.[2,3] The study by Kotler *et al*[2] investigated the exon III VNTR in 141 male opioid-dependent subjects and 110 male control subjects from Israel, and found a significant excess of the 7-repeat allele, with an odds ratio of 3.06 (95% CI 1.38–4.35). Li *et al*[3] studied a Chinese sample of 121 heroin-dependent subjects and 154 controls. While the 7-repeat allele is extremely rare in Chinese populations and was only observed in two patients, dividing the VNTR alleles into 'long' (5–7 repeats) and 'short' (2–4 repeats) alleles showed a significant excess of long alleles in the patient group, with an odds ratio of 2.30 (95% CI 1.07–4.93). These observations clearly suggest a causal contribution of genetic variation in the DRD4 to the development of opioid dependence. In the present study, we have attempted to replicate this finding by genotyping DNA samples from 285 heroin addicts and 197 controls. Since a major flaw of case-control association studies is the problem of undetected population stratification, we also applied a family-based association study design by genotyping an independent sample of 111 heroin addicts and their parents to examine preferential transmission of alleles to affected offspring.

The distributions of genotype and allele frequencies of the DRD4 exon III VNTR in patients and controls are shown in Tables 1 and 2. There were no significant differences between patients and controls in the frequency of the 7-repeat allele ($\chi^2 = 2.444$; df = 1; $P = 0.188$) nor in any of the other alleles (Table 2). At least one 7-repeat allele was found in 35.1% of heroin-dependent subjects and 27.4% of controls ($\chi^2 = 3.157$; df = 1; $P = 0.076$). Moreover, patients and controls did

Table 1 Distribution of genotypes of the DRD4 exon III VNTR polymorphism in opioid-dependent patients and control individuals. Genotype frequencies are given in brackets

Genotype	*Heroin-dependent patients*	*Controls*
2–2	4 (1.4%)	1 (0.5%)
2–3	0 (0%)	1 (0.5%)
2–4	29 (10.2%)	22 (11.2%)
2–7	13 (4.6%)	3 (1.5%)
2–8	1 (0.4%)	0 (0%)
3–3	2 (0.7%)	0 (0%)
3–4	21 (7.4%)	11 (5.6%)
3–5	1 (0.4%)	0 (0%)
3–7	4 (1.4%)	1 (0.5%)
4–4	115 (40.4%)	94 (47.7%)
4–5	2 (0.7%)	10 (5.1%)
4–6	2 (0.7%)	2 (1.0%)
4–7	72 (25.3%)	42 (21.3%)
4–8	7 (2.5%)	1 (0.5%)
4–9	1 (0.4%)	0 (0%)
5–5	0 (0%)	1 (0.5%)
5–7	1 (0.4%)	1 (0.5%)
7–7	9 (3.2%)	6 (3.1%)
7–8	1 (0.4%)	1 (0.5%)
Total	285	197

Table 2 Allele distribution of the DRD4 exon III VNTR polymorphism in opioid-dependent patients and control individuals. Allele frequencies are given in brackets

Allele	*Heroin-dependent patients*	*Controls*
2	51 (8.9%)	28 (7.1%)
3	30 (5.3%)	13 (3.3%)
4	364 (63.9%)	276 (70.1%)
5	4 (0.7%)	13 (3.3%)
6	2 (0.4%)	2 (0.5%)
7	109 (19.1%)	60 (15.2%)
8	9 (1.6%)	2 (0.5%)
9	1 (0.2%)	0 (0%)
Total	570	394

not differ significantly in frequency of individual genotypes (Table 1) (χ^2 = 26.180; df = 18; P = 0.096), or in the frequency of short (2–4 repeats) *vs* long (5–8) alleles (χ^2 = 0.801; df = 1; P = 0.371). Similar negative results were obtained when the analysis was limited to male subjects (data not shown).

Using the transmission disequilibrium test (TDT)[7] in the 111 parent-offspring trios, no preferential transmission of either the 7-repeat allele *vs* non-7 alleles (TDT = 0.111; P = 0.739) or long alleles *vs* short alleles (TDT = 0.409; P = 0.522) were observed (Table 3).

In complex disorders, such as substance abuse, which result from the interaction of various vulnerability genes, association studies are the most efficient strategy to explore the putative contribution at candidate gene loci.[8,9] Not withstanding the power of association studies in detecting small gene effects, the main methodological problem with all conventional case-control studies is the implicit assumption that cases and controls are both from the identical genetic population. Since allele frequencies often vary considerably between populations, as has been demonstrated for the DRD4 exon III VNTR,[10] minor biases in sampling of patients and controls can produce significant stratification effects. These could either produce a false positive finding or obscure a true positive result. Hence, a recent alternative to traditional case-control association analyses are allele-based, within-family analyses, in which population stratification due to ethnicity is controlled due to both case and control alleles coming from the same family.[7,11,12]

Table 3 Transmission of DRD4 exon III VNTR alleles to opioid-dependent offspring

Transmitted alleles →	*Non-transmitted alleles*							
	2	*3*	*4*	*5*	*6*	*7*	*8*	*Total*
2	0	1	14	0	0	3	0	18
3	2	0	3	0	0	1	0	6
4	11	7	0	0	0	35	2	55
5	0	0	3	0	0	0	0	3
6	0	0	2	0	0	0	0	2
7	7	1	33	0	0	0	1	42
8	0	0	1	0	0	0	0	1
Total	20	9	56	0	0	39	3	127

Allele 7 *vs* other alleles: TDT = 0.1111 (P = 0.739).
Short alleles (2–5) *vs* long alleles (6–8): TDT = 0.4091 (P = 0.522).

Based on these considerations, we applied a two-tiered strategy employing ethnically matched case-control as well as family-based association samples to evaluate a possible contribution of the DRD4 exon III VNTR to the development of opioid dependence. Our overall data clearly provide no positive evidence to support a role for DRD4 in the German population. Our inability to detect an effect does not appear to be secondary to insufficient power. Power calculations demonstrate a power of >99% for the case-control sample and of 98% for the family-based association sample to detect an effect of the magnitude reported by Kotler *et al*.[2] The lack of supportive data from our study suggests that any relevance that might exist is much smaller than previously suggested.

The present results are in agreement with a recent case-control association study by Gelernter *et al*[13] who studied DRD4 exon III genotypes in a mixed sample of 308 substance abusers, with a high proportion of alcoholics, and 144 controls. However, due to the phenotypic and ethnic heterogeneity of this sample, the conclusion of no effect of the DRD4 exon III VNTR on opioid dependence would be problematic. Most likely, the previous reports represent false positive findings which may have been caused by population stratification or simply by chance. However, other possible reasons for our non-replication have to be considered. Although the exon III VNTR has been shown to affect protein function,[14–16] it is a possibility that the exon III VNTR is not itself the causally relevant mutation and that a population-specific mutation conveying an affect on opioid dependence is in close linkage disequilibrium with the VNTR, although no data exist to support this hypothesis. The coding region of the human DRD4 gene has been the subject of extensive mutation screening and no variant, which could account for the effect through linkage disequilibrium, has been identified to date.[14,17–21] However, since the mutation screenings were performed in other than Israelian and Chinese populations it cannot be excluded that population-specific variants exist. It may also be possible that population-specific variation is present in regulatory regions of the gene, which still have to be defined at the molecular level. Finally, it remains possible that the DRD4 exon III VNTR is acting only in conjunction with other (as yet undefined) population-specific genetic or environmental factors.

In conclusion, we failed to replicate previous reports of an association between long alleles of the DRD4 exon III VNTR and opioid dependence. Our finding was based on the investigation of two independent samples: a large ethnically matched case-control

association sample and a sample of parent-offspring trios which was used for family-based association analysis. Either sample had a high power to detect an effect of the reported size. Our lack of supportive data questions the role of the DRD4 exon III VNTR in susceptibility to opioid dependence.

Materials and methods

The samples

Three hundred and ninety-six heroin-dependent patients (mean age: 30.8 years; mean age-at-onset of heroin dependence: 21.5 years) were recruited from consecutive admissions to the Opioid Detoxification Unit of the Department of Psychiatry, University of Bonn. All patients were interviewed using a semi-structured interview for axis I and II disorders and all fulfilled DSM-III-R criteria for opioid dependence (97.3% of the patients fulfilled five or more DSM-III-R criteria). Written informed consent was obtained from all patients participating in this study. Of 111 heroin addicts, both biological parents were willing to participate in the study. These parent-offspring trios formed the family-based association sample. The patients with no or only one available parent were evaluated in the case-control sample, which finally included 285 heroin-dependent patients. Patients with and without two available parents did not differ in demographic characteristics or addiction severity from each other. One hundred and ninety-seven probands (mean age: 30.0 years) were taken as controls and have been described elsewhere in detail.[22] All patients and controls were of German origin.

The study was approved by the local ethical committee of the University of Bonn, Germany.

Genotype analysis

Venous blood samples, anticoagulated with EDTA, were drawn from all individuals. Genomic DNA was isolated from peripheral blood lymphocytes by a standard salting-out method. Analysis of the 48-bp repeat polymorphism in exon III of the DRD4 gene was done as described by Lichter *et al*.[18]

Statistical analysis

We applied χ^2 test for comparing genotype distributions and allele frequencies between cases and controls. A power analysis was calculated for the case-control sample based on the given odds ratio (OR) of allele 7 (genotype 7/7 or 7/x) by Kotler *et al*.[2] The power of our case-control sample was >99%. The calculation was based on a frequency of allele 7 of 0.12 and 0.27.[2,22] The *a posteriori* power (parental mating type) in our family-based sample of 111 heroin-dependent patients was 0.98, to detect genotype-associated relative risks observed in the previous study.

The transmission disequilibrium test (TDT)[7] was applied for analysis of parent-offspring trios. The TDT is based on the detection of disproportionate transmission of high- *vs* low-risk alleles by heterozygous parents to affected children. The Mendelian expectation under the null hypothesis of no association is that either allele carried by a heterozygote has a 50:50 chance of transmission to an affected child. If the allele plays a causal role in the development of the disorder, however, then its transmission should exceed 50%. The statistical significance level was set at $P = 0.05$.

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