American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 125B:20–24 (2004)

No Evidence of Association or Linkage Disequilibrium Between Polymorphisms in the 5′ Upstream and Coding Regions of the Dopamine D4 Receptor Gene and Schizophrenia in a Portuguese Population

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Alterations in dopaminergic system have been implicated in the pathophysiology of this disease for many years, and this study was performed to assess the possible involvement of the dopamine D4 receptor (*DRD4*) gene polymorphisms either in the 5′ upstream or in the coding regions, in the etiology of schizophrenia. The approach included an association study with 90 Portuguese trios by doing the analysis of the individual alleles and the haplotypes. For the polymorphisms in the 5′ upstream region (−C616G and −C521T) and in the coding region (48 bp repeat) of the *DRD4* gene, negative results were obtained with both haplotype relative risk (HRR) and transmission disequilibrium test (TDT), as well as transmit. These data suggest that polymorphisms (−C616G, −C521T, and 48 bp repeat) at the *DRD4* gene do not have a minor effect in the susceptibility to schizophrenia in our sample.

KEY WORDS: schizophrenia; association study; dopamine D4 receptor; candidate gene; linkage disequilibrium; haplotypes

INTRODUCTION

Schizophrenia is a clinically heterogeneous disease involving genetic and environmental factors. Despite extensive research efforts, no mutations, or disease predisposing DNA sequence variations, have been identified, and the molecular basis of genetics remain elusive. Although several linkage studies have been performed to localize major effect susceptibility genes for schizophrenia [Riley and McGuffin, 2000; Baron, 2001], association studies are also relevant, since the genetic etiology may be multifactorial or polygenic. Therefore, any single susceptibility gene contributes only a small fraction to the overall risk, and allelic variation at such genes must be high, and can be directly evaluated as susceptibility factors using candidate gene association studies [Risch and Merikangas, 1996]. Indeed, linkage disequilibrium mapping is a powerful alternative to linkage analysis for the mapping and detection of genes involved in schizophrenia with a modest effect [Lander, 1996; Risch and Merikangas, 1996; Collins et al., 1997; Ott and Hoh, 2000].

Disturbances in dopamine neurotransmission and dopamine receptors have long been suggested to play a crucial role in the pathogenesis of schizophrenia [Prasad et al., 2002]. To date, five distinct subtypes of G protein coupled dopamine receptors mediate the actions of dopamine (DRD1, DRD2, DRD3 DRD4, and DRD5) (for review see Missale et al., 1998). The gene which

Grant sponsor: Medical Research Council of Canada; Grant sponsor: National Institute of the Mental Health of USA; Grant sponsor: Junta Nacional de Investigação Científica e Tecnológica, Portugal.

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Received 5 February 2003; Accepted 5 June 2003
DOI 10.1002/ajmg.b.20084

codes for dopamine receptor D4 (DRD4) is considered as one of the most relevant candidate genes of schizophrenia because it is expressed in limbic system and clozapine, the prototype of atypical antipsychotics, binds to DRD4 with an affinity ten times higher than to DRD2 and DRD3 receptors [Van Tol et al., 1991]. In addition, *DRD4* gene shows a unique high degree of variation in the human population [Van Tol et al., 1992], with several polymorphisms located both in translated and non-translated regions [Van Tol et al., 1992; Lichter et al., 1993; Petronis et al., 1994; Paterson et al., 1996], with a particular relevance for variants in the 5′ upupstream region [Seaman et al., 1999; Barr et al., 2001]. Although several polymorphisms have been described in the *DRD4* gene, the most widely studied in schizophrenia, was the polymorphism 48 bp repeat localized in the third exon [Lichter et al., 1993], with conflicting results, potentially because of the differences of methodology [Barr et al., 1993; Kennedy et al., 1993; Sommer et al., 1993; Macciardi et al., 1994; Shaikh et al., 1994; Petronis et al., 1995; Skaikh et al., 1995; Tanaka et al., 1995; Paterson et al., 1996; Serretti et al., 2001]. To solve these conflicting results, more independent association studies are required, using a homogeneous population, as for example the Portuguese population [Schindler et al., 1999]. In small homogeneous populations, the genetic variability can be reduced and the environmental and cultural variabilities are, likewise, lower than cosmopolitan population. These factors should increase the chances of detecting at least some possible genetic loci. Our group has been studying the genetics of psychotic disorders in a sample with reduced heterogeneity derived from mainland Portugal and Azores, Portugal. The Azores are a nine-island archipelago in the Atlantic settled exclusively by the Portuguese in the early 1400s. The familial transmission has been consistent and well documented for at least 300 years. The average number of families with the same last name is a good marker for the degree of homogeneity. Surname analysis of the population showed a 79-fold reduction in variability in surnames for the Azorean population and a 55-fold reduction in surnames for the Continental Portuguese population, in comparison to United States population [Schindler et al., 1999]. Furthermore, it is important to investigate polymorphisms in the 5′ upstream region, because variations in the regulation of the gene could be more important than any structural changes in the protein. Thus, the aim of the present study was to investigate the possible involvement of several polymorphisms of the *DRD4* gene in the etiology of schizophrenia, particularly −C616G and −C521T polymorphisms in the region 5′, at the start site of transcription, and the 48 bp repeat polymorphism, in the coding region.

MATERIALS AND METHODS

Patient Sample

For this study, the sample consists of 90 unrelated schizophrenic patients from Azores and Mainland, Portugal (58 males and 32 females) and their parents, a total of 270 individuals. Approval was obtained from local ethics committees and informed consent was taken from all subjects. All patients were personally interviewed using the Diagnostic Interview for Genetics Studies (DIGS), rated with the Operational Criteria checklist OPCRIT, and all were diagnosed according to DSM-IV.

Genetic Analysis

High-molecular weight genomic DNA was extracted from peripheral blood leukocytes according to the standard method described by Miller et al. [1988], with slight modifications.

Three polymorphisms in the *DRD4* gene were investigated, two in the 5′ upstream region (−C616G and −C521T) and one in the coding region (48 bp repeat). PCR amplification of the polymorphisms (−C616G and −C521T) of the *DRD4* gene was achieved as described by Barr et al. [2001]. Briefly, PCR amplification was carried out in a final volume 20 μl, with the PCRx enhancer system (1 × PCR amplification buffer, 1 × PCRx enhancer solution), 100 ng genomic DNA template, 200 μM dNTPs, 5 ng/μl of each primer, 1.5 mM $MgCl_2$, and 0.5 U of Taq polymerase (Perkin Elmer, Toronto, Canada). After an initial denaturation step at 94°C for 4 min, there were 35 cycles, each consisting of 94°C for 40 sec, 58°C for 40 sec, and 72°C for 40 sec. PCR products (7 μl for each polymorphism) were digested using either enzyme AvaII or BssHI, and the digested fragments were separated in 2.0 and 2.5% agarose gel, respectively, and were visualized by ethidium bromide staining.

The 48 bp repeat polymorphism localized in third exon of *DRD4* gene was detected by PCR according to the method descrobed by Lichter et al. [1993]. The PCR was carried out in a total volume of 25 μl containing 200 ng genomic DNA as template, 200 μM (dATP, dTTP, dCTP), 100 μM dGTP, 100μM deaza-dGTP, 1.0 μM of each primer, 10 mM Tris-HCl (pH = 8.3), 1.0 mM $MgCl_2$, 10% DMSO, and 1 U of Taq polymerase (Perkin-Elmer). PCR amplification is initiated at 95°C for 5 min and performed for 40 cycles of 95°C for 20 sec, 54°C for 20 sec, and 72°C for 40 sec. Fragments were separated in 3.5% agarose gel and visualized by ethidium bromide.

Statistical Analysis

Statistical analysis was performed using the haplotype relative risk (HRR) [Terwilliger and Ott, 1992], and transmission disequilibrium test (TDT) [Spielman et al., 1993; Spielman and Ewens, 1998] which avoid any potential population stratification. We used the HRR and TDT to test if the marker locus and the hypothetical disease locus were linked or in linkage disequilibrium. The TDT–STDT programe (v 1.1) uses data from heterozygous parents only, and test for individual markers. Furthermore, multiple marker haplotype transmission was performed with program TRANSMIT v 2.5 [Clayton, 1999]. The program TRANSMIT tests for association between markers and disease, examining the transmission of multilocus haplotypes.

TABLE I. HRR and TDT Results for (−C616G) Polymorphism of *DRD4* Gene in Portuguese Schizophrenic Trios

		DRD4 gene		
		Transmitted	Not transmitted	χ^2 (df = 1), *P* value
HRR	C616	113	110	$\chi^2 = 0.047$, $P = 0.828$
	G616	67	70	
TDT	C616	54	49	$\chi^2 = 0.243$, $P = 0.622$
	G616	49	54	

TABLE II. HRR and TDT Results for (−C521T) Polymorphism of *DRD4* Gene in Portuguese Schizophrenic Trios

		DRD4 gene		
		Transmitted	Not transmitted	χ^2 (df = 1), *P* value
HRR	C521	85	93	$\chi^2 = 0.545$, $P = 0.461$
	T521	95	87	
TDT	C521	57	63	$\chi^2 = 0.300$, $P = 0.584$
	T521	63	57	

RESULTS

We have performed an association study with 90 trios and −C616G and −C521T polymorphisms in the upstream region, as well as a 48 bp repeat polymorphism in the coding region in the *DRD4* gene. Tables I and II show the results of HRR and TDT analysis for the −C616G and −C521T polymorphisms in the *DRD4* gene. Using the HRR and TDT methods, no significant differences were observed between transmitted and not transmitted alleles for both polymorphisms in the 5′ upstream region (−C616G polymorphism $\chi^2 = 0.047$, df = 1, $P = 0.828$; −C521T polymorphism $\chi^2 = 0.545$, df = 1, $P = 0.461$). Concerning 48 bp repeat polymorphism of the *DRD4* gene the allele frequencies were four repeats >7 repeat >2 repeats > rare alleles. Similarly, HRR and TDT analysis yielded non-significant *P* values for association of 48 bp repeat polymorphism at the *DRD4* gene with schizophrenia ($\chi^2 = 0.108$, df = 3, $P = 1.000$; Table III). Furthermore, we tested the transmission of alleles from −C616G, −C521T, and 48 bp repeat polymorphisms as a haplotype using TRANSMIT and the results are shown in Table IV. The analysis of multiple marker haplotypes provided no evidence for association with schizophrenia at this locus. Also, we estimated the haplotype frequencies of three markers in the *DRD4* gene, and a total of 16 haplotypes were detected, but Table IV presents the haplotypes with a frequency above 3% only.

DISCUSSION

Schizophrenia is one of the most extensively studied brain disorders in molecular genetic studies, and primordial attention was given to dopaminergic system in the etiology of this disorder, with special focus on the dopamine D2-like (*DRD2*, *DRD3*, *DRD4*) receptor subtype genes, with inconclusive results. In complex disorders, like schizophrenia, which result from various vulnerability genes, family association studies are the most appropriate approaches to explore the putative contribution at candidate gene loci [Ott and Hoh, 2000]. Several factors could complicate the molecular genetics of schizophrenia including genetic heterogeneity. In order to avoid this factor, it is extremely crucial to study homogenous populations, as for example the Portuguese population. Whereas most studies have investigated only the 48 bp repeat in the third exon, which alters the third intercytoplasmic loop, it is important to investigate polymorphisms in the 5′ upstream region, because variations in the regulation of the gene could be more important than any structural changes in the protein. Thus, in this study we investigated two polymorphisms in the 5′ upstream region (−C616G and −C521T) and one polymorphism in the coding region (48 bp repeat) at *DRD4* gene for susceptibility to schizophrenia in the Portuguese population by using family-based association analysis. In the present study, using a dual genetic approach of association analysis, HRR and TDT, no evidence for association or linkage disequilibrium was found for the individual alleles or the haplotypes of the polymorphisms −C616G, −C521T, and 48 bp repeat, of the *DRD4* gene and schizophrenia. The results of this study indicate that, in our sample, the −C616G and −C521T polymorphisms are not directly involved in the pathogenesis of schizophrenia, and are in agreement with an association study that found no association between both polymorphisms and schizophrenia [Mitsuyasu et al., 2001]. Concerning the 48 bp repeat polymorphism of the *DRD4* gene, our finding is different from previous case-controls association study in the

TABLE III. HRR and TDT Results for 48 bp Repeat Polymorphism of *DRD4* Gene in Portuguese Schizophrenic Trios

		DRD4 gene		
		Transmitted	Not transmitted	χ^2 (df = 3), *P* value
HRR	2 Repeat	23	21	$\chi^2 = 0.108$, $P = 1.000$
	4 Repeat	115	117	
	7 Repeat	39	39	
	Others	3	3	
TDT	2 Repeat	18	15	$\chi^2 = 0.273$, $P = 0.642$
	4 Repeat	46	48	$\chi^2 = 0.043$, $P = 0.836$
	7 Repeat	34	35	$\chi^2 = 0.014$, $P = 0.906$
	Others	2	2	$\chi^2 = 1.000$, $P = 0.317$

TABLE IV. Estimated Haplotype Probabilities and Chi Squared Test of Transmission of Multi-Marker Haplotypes at the *DRD4* Gene Using TRANSMIT

	DRD4 gene				
Haplotypes	Observed	Expected	O-E	Frequency	Haplotypic *P* value
2-1-1	6.111	7.295	2.392	0.041	0.444
4-1-1	33.162	33.104	12.840	0.183	0.987
7-1-1	18.399	16.960	5.654	0.094	0.545
4-2-1	18.552	22.300	7.296	0.123	0.165
2-1-2	7.001	6.803	1.859	0.037	0.884
4-1-2	33.526	32.381	13.915	0.179	0.759
7-1-2	10.409	10.259	3.449	0.056	0.935
4-2-2	29.761	28.214	11.661	0.156	0.651
7-2-2	6.532	8.066	2.896	0.044	0.367

Portuguese population [Valente et al., 1997], as well as in another population [Okuyama et al., 1999], where a positive association between *DRD4* gene and schizophrenia was found. The failure of our study to replicate the positive association between the 48 bp repeat polymorphism and schizophrenia in a Portuguese population may have been due to a stratification effect, and that positive association might have been due to a chance positive finding. However, our results are in agreement with other association studies that found no association between the *DRD4* gene and schizophrenia [Sommer et al., 1993; Petronis et al., 1995; Skaikh et al., 1995; Tanaka et al., 1995; Paterson et al., 1996; Serretti et al., 2001], and also in accordance with family association and linkage studies that found no association and no linkage between schizophrenia and DRD4 [Barr et al., 1993; Kennedy et al., 1993; Macciardi et al., 1994; Shaikh et al., 1994].

Despite these results, the dopamine system remains an important area of investigation in schizophrenia. Indeed, the identification of genes that contribute to the susceptibility to schizophrenia has been disappointing using traditional genetic approaches. Further directions to identify the susceptibility genes include the use of larger samples in order to increase the statistical power, the development of more powerful statistical analysis applying a best definition of the clinical phenotype of the disease by using endophenotypes, and performing advanced molecular genetic techniques [Ott and Hoh, 2000; Baron, 2001; Cornblatt and Malhotra, 2001; Gordon et al., 2001; Hoh and Ott, 2001; Hoh et al., 2001; Majewski et al., 2001; Tsuang, 2001].

In summary, using two different association methodologies (HRR and TDT) as well as TRANSMIT both studies were unable to demonstrate a minor effect of the *DRD4* gene in the predisposition to schizophrenia in our family data. The clinical manifestations of schizophrenia suggest a disease produced by a variable mixture of genetic predispositions. Therefore, we may conclude that dopamine hypothesis of schizophrenia by its own is clearly insufficient to explain many biological factors underling this disorder, and the involvement of dopamine in schizophrenia together with other neurotransmitter changes is more crucial.

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