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NR4A2 and Schizophrenia: Lack of Association in a Portuguese/Brazilian Study

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The present study investigates the association of mutations in the nuclear receptor NR4A2 in schizophrenic patients. The human Nur-related receptor 1, NR4A2, is an orphan nuclear receptor that can be constitutively active as a transcription factor and for which no natural ligand has yet been identified. Alone or with retinoid X receptor, RXR, NR4A2 influences the expression of several genes important for human brain development and regulation. In the absence of Nurr1 (the mouse homologue to human NR4A2), ventral mesencephalic dopaminergic mouse neurons evidence severe developmental failure, a condition that is lethal soon after birth. Nurr1 involvement in the dopaminergic system makes it a good candidate for study in neuropsychiatric disorders such as schizophrenia and Parkinson disease. Evidence by others support this hypothesis (1) mapping of the *NR4A2* gene to chromosome 2q22-23, a region with suggestive linkage to schizophrenia and (2) identification of mutations in patients with schizophrenia (c.366-369delTAC, c.308A > G, c.-469delG), manic depression (c.289A > G), and familial Parkinson's disease (c.-291delT, c.-245T > G). To further extend these observations, we searched for all these mutations in 176 Caucasian Portuguese and 82 Caucasian Brazilian subjects with lifetime diagnosis of schizophrenia. The study failed to identify any of the described mutations in patients or controls. Nevertheless, these negative results do not exclude altered expression of nuclear receptors in schizophrenia or the presence of other mutations.

KEY WORDS: Nurr1; nuclear orphan receptor; dopamine; retinoids

INTRODUCTION

Schizophrenia is a chronic psychotic disorder of unknown etiology. Twin and family studies demonstrate a genetic basis for the disease [Tsuang et al., 2001]. Several genome wide studies identified chromosome loci consistently linked to increased susceptibility to schizophrenia in different populations [Lewis et al., 2003]. Despite the evidence for genetic predisposition, the lack of concordance in monozygotic twins implies that an epigenetic environmental effect is required for disease onset [Tsuang et al., 2001]. For this reason, interest has been raised in hypothesis bridging both genetic and environment-related mechanisms. Environmental factors such as hormones and vitamins interact with different nuclear receptors and interfere with the transcription of several genes on a developmental regulated fashion. Among these are the retinoids, considered good candidates because of genetic linkage studies implicating schizophrenia with disregulation of the retinoid cascade and/or genes whose expression they regulate [Goodman, 1998]. The genes for dopamine-2 receptor, synapsin, and dopamine β-hydroxylase are among those whose expression requires activation by retinoids [Samad et al., 1997; Kim et al., 2001, 2003; Balmer and Blomhoff, 2002]. Pharmacological, histological, and brain imaging data have for long implicated dopaminergic dysfunction in the etiology of schizophrenia. Retinoid availability can, therefore, interfere with susceptibility to schizophrenia, either directly, through the dopamine system, or indirectly through molecules involved in its development and/or regulation. Interestingly, retinoid analogs have been suggested in the treatment of schizophrenia, alone or in combination with dopamine receptor agonists [Citver et al., 2002].

Retinoic acid receptors belong to the steroid/thyroid hormone nuclear receptor superfamily that includes several orphan receptors for which no ligands have been identified. These receptors often act as heterodimers greatly increasing the complexity of gene regulation. NR4A2, one of these orphan nuclear receptors, is known to regulate transcription of target genes in two different ways: alone, as a monomer, or as a partner with the retinoid X receptor (RXR) [Perlmann and Jansson, 1995; Wang et al., 2003].

Nurr1 (the mouse homologue to human *NR4A2* gene) mRNA is expressed very early in the ventral midbrain [Zetterström et al., 1996, 1997] and disruption of its expression is responsible for massive failure to generate dopaminergic neurons in the midbrain and causes death soon after birth [Zetterström

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et al., 1997; Saucedo-Cardenas et al., 1998]. The lack of dopaminergic neurons in the midbrain seems to be related with the inability to express tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis [Zetterström et al., 1996]. Furthermore, Nurr 1 seems to retain its role in mature dopaminergic neurons, since its expression continues over adulthood and is extremely impaired after ventral mesencephalic dopaminergic neurons injury [Zetterström et al., 1996].

These observations increased the interest in identifying precise molecular and biochemical mechanisms of behavior regulation by NR4A2 in psychiatric and neurological disorders. Furthermore, the 2q22-q23 chromosome region that harbors the *NR4A2* gene has suggestive linkage with schizophrenia in various populations [Moises et al., 1995; Williams et al., 1999; DeLisi et al., 2002; Lewis et al., 2003]. In accordance to this hypothesis several groups have looked for mutations in the *NR4A2* human gene that could increase susceptibility to schizophrenia. Up to date, two deletions and one missense mutation have been described in schizophrenic patients and one missense mutation has been found in manic-depressed individuals [Buervenich et al., 2000; Chen et al., 2001] while, recently, two mutations in the untranslated region have been identified in patients with familial Parkinson's disease [Le et al., 2003]. These findings prompted us to investigate all these NR4A2 mutations in a large sample of Portuguese and Brazilian schizophrenic patients. Patients fulfilled DSM IV lifetime diagnosis of schizophrenia, confirmed by Operational Checklist for Psychotic Disorders (OPCRIT) diagnostic algorithm. None of the mutations described in the literature were identified in this group of patients.

MATERIALS AND METHODS

The sample consisted in 176 Portuguese (age range 15–74, average 34.0; 67 female and 109 male) and 82 Brazilian (age range 18–63, average 34.3; 10 female and 72 male) schizophrenic patients and 105 Portuguese (age range 19–79, average 35.5; 53 female and 52 male) and 85 Brazilian (age range 19–58, average 32.9; all men) mentally healthy individuals. All subjects were unrelated Caucasians. Patients gave informed consent for the study, and ethic committees of both institutions approved the study. All patients (Portuguese and Brazilian) were classified by the OPCRIT system [McGuffin and Farmer, 2001]. OPCRIT automated system gathered information from all case records, including medical, nursing, social work and occupational therapy notes together with data from clinical interview with patient and relatives. Additionally, Portuguese schizophrenic patients received lifetime diagnosis using DIGS (Diagnostic Interview for Genetic Studies) as previously described [Nurnberger et al., 1994]. Controls were from European origin or descent and free of any lifetime diagnosis of major mental illness and physical illness. Portuguese controls received DIGS assessment and Brazilian controls were selected among blood bank volunteer donors documented to be free of chemical dependence [Rios et al., 2003]. All interviews and diagnostic formulations were performed by one of the authors. Venous blood was drawn from all subjects and DNA extracted using standard salting-out procedures.

For genotyping the c.-245T > G, c.289 A > G, c.308 A > G mutations and the deletion c.-469delG, we followed PCR conditions previously described [Buervenich et al., 2000; Chen et al., 2001; Le et al., 2003]. PCR products were analyzed on gel electrophoresis after digestion with the restriction enzymes *Ava II* (Fermentas, Vilnius, Lithuania), *ApaL* I (Fermentas), *Tse* I (New England BioLabs, Beverly, MA), and *Cfr* I (Fermentas), respectively. The c.366-369delTAC deletion was screened by single strand conformation polymorphism (SSCP) on the PCR product amplified with the primers (5′-3′) CTTGTACCAAATGCCCCTGT and GAGACTGGCGTTTT-CCTCT. Electrophoresis on 10% non-denaturing polyacrylamide gel with 2.5% glycerol was performed at 500 V for 2.5–3 hr. Temperature was strictly maintained at 14°C. Samples from patient carriers of the mutations c.366-369delTAC, c.289 A > G, and c.308 A > G were used as controls.

Search for the c.-291delT on the PCR product amplified with primers previously described [Le et al., 2003] was done by SSCP analysis on MDE gel (Cambrex Bio Science Rockland, Rockland, ME) run at 4, 10, or 25°C.

RESULTS

Using SSCP analysis, we searched the deletions c.366-369delTAC and c.-291delT, looking also for other possible mutations [Vidal-Puig and Moller, 1994] on the same PCR product. Figure 1 shows the SSCP migration pattern of a c.366-369delTAC deletion carrier. None of the sample tested revealed the presence of mutated alleles.

For the c.-291delT deletion we analyzed one subpopulation of 60 Portuguese schizophrenic patients and run the SSCP at three different temperatures to increase the rate of detection. Again, we failed to identify any mutation in the PCR product containing the position c.-291. Analysis of the mutations c.-469delG, c.-245T > G, c.289A > G, and c.308A > G was done by digestion with the enzymes *Cfr* I, *Ava* II, *ApaL* I, and *Tse* I for which a new restriction site is present in the mutated allele. None of the samples, from schizophrenic or mentally healthy individuals, contained any of the mutations screened.

Table I is a summary of all studies, including ours, in which NR4A2 mutations described in diseases have been investigated.

DISCUSSION

In the present study we investigated, for the first time, the presence of two NR4A2 mutations recently identified in familial Parkinson's disease [Le et al., 2003] in 258 schizophrenic patients and 190 mentally healthy individuals. We also searched for mutations in the NR4A2 previously described in patients with schizophrenia and manic-depression [Buervenich et al., 2000; Chen et al., 2001]. No mutation was found either in patients or controls. Given the complex etiology of schizophrenia and the failure to identify a single causative gene, it is unlikely that any individual mutation will be strongly represented in the patient's population [Chakravarti, 1999]. In the case of transcription factors such as nuclear receptors, several different mutations can impair proper function and influence the appropriate expression of several genes. Therefore, for any new mutation found it is important to increase the size of the patient sample analyzed. The fact that

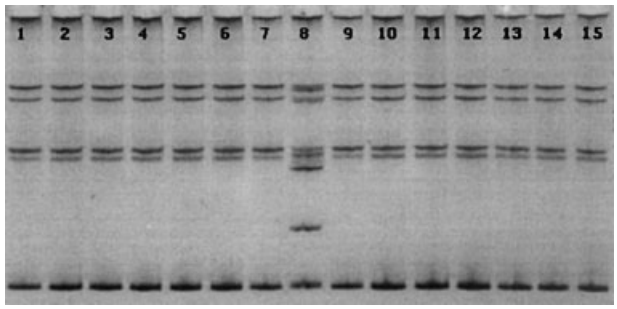

Fig. 1. Single strand conformation polymorphism (SSCP) analysis of the NR4A2 exon 3 PCR fragment. On **lane 8** run a sample containing the c.366-369delTAC mutation. **Lanes 1–6** correspond to Brazilian schizophrenic patients and **lanes 7, 9–15** to Brazilian mentally healthy individuals.

TABLE I. NR4A2 Mutations Detected in Patients With Schizophrenia, Manic Depression, and Parkinson's Disease

Mutation	Location	Population	In vitro transcription activity
c.-469 delG	Promoter	2/176 Han Chinese schizophrenic [Chen et al., 2001] 0/176 Caucasian Portuguese schizophrenic 0/82 Caucasian Brasillan schizophrenic	Not determined
c.-291 delT	Exon 1, untranslated	8/107 familial + 0/94 sporadic Parkinson's (mostly Caucasian) [Le et al., 2003] 0/60 Caucasian Portuguese schizophrenic	Decreased [Le et al., 2003]
c.-245 T > G	Exon 1, untranslated	2/107 familial + 0/94 sporadic Parkinson's (mostly Caucasian) [Le et al., 2003] 0/176 Caucasian Portuguese schizophrenic 0/82 Caucasian Brasilian schizophrenic	Decreased [Le et al., 2003]
c.289 A > G	Exon 3, coding	0/135 Swedish schizophrenics [Buervenich et al., 2000] 0/160 North American Caucasian schizophrenics [Buervenich et al., 2000] 0/70 Caucasian Swedish idiopathic Parkinson [Buervenich et al., 2000] 1/30 Caucasian Swedish manic depressed [Buervenich et al., 2000] 0/176 Caucasian Portuguese schizophrenic 0/82 Caucasian Brasilian schizophrenic	Decreased [Buervenich et al., 2000]
c.308 A > G	Exon 3, coding	1/135 Swedish schizophrenics [Buervenich et al., 2000] 0/160 Caucasian North American schizophrenics [Buervenich et al., 2000] 0/70 Caucasian Swedish Idiopathic Parkinson [Buervenich et al., 2000] 0/30 Caucasian Swedish manic depressed [Buervenich et al., 2000] 0/176 Caucasian Portuguese schizophrenic 0/82 Caucasian Brasilian schizophrenic	Decreased [Buervenich et al., 2000]
c.366-369 del TAC	Exon 3, coding	1 childhood-onset/135 Caucasian Swedish schizophrenics [Buervenich et al., 2000] 0/160 Caucasian North American schizophrenics [Buervenich et al., 2000] 0/70 Caucasian Swedish Idiopathic Parkinson [Buervenich et al., 2000] 0/30 Caucasian Swedish manic depressed [Buervenich et al., 2000] 0/176 Caucasian Portuguese schizophrenic 0/82 Caucasian Brasilian schizophrenic	Decreased [Buervenich et al., 2000]

Represented is the number of individuals in which NR4A2 mutations were found among all patients tested.

we failed to identify the mutations previously described confirms that they are very rare in *NR4A2* gene.

Other studies have reported lack of association of NR4A2 polymorphic variants in the promoter [Carmine et al., 2003], 5′and 3′ untranslated regions and intron 6 with schizophrenia [Ishiguro et al., 2002; Iwayama-Shigeno et al., 2003].

The human *NR4A2* gene exists as a single locus in human genome, covering 8.3 Kb in length, consisting of eight exons and is mapped to chromosome 2q22-23 [Ichinose et al., 1999; Torii et al., 1999]. This chromosome region has been implicated in schizophrenia [Lewis et al., 2003]. The mutations c.289A > G, c.308A > G, and c.366-369delTAC, originate amino acid changes at the protein level (M97V, H103R, and ΔY122, respectively) and result in decreased in vitro transcriptional activity of NR4A2 dimmers [Buervenich et al., 2000]. Decreased transcription activity of the NR4A2 is also described for the mutations in the 5′ untranslated region found in patients with Parkinson's disease [Le et al., 2003]. Therefore, impaired transcription activation of downstream target genes such as tyrosine hydroxylase is expected in carriers of these mutant variants of NR4A2.

Studies in mice have revealed important functions for NR4A2 that clearly suggest its possible involvement in several disorders of the central nervous system in which the dopaminergic system has been implicated. Observations in Nurr1-null mice revealed that Nurr1 is requested for the formation of midbrain dopaminergic neurons [Zetterström et al., 1997; Saucedo-Cardenas et al., 1998] and that tyrosine hydroxylase, the rate-limiting enzyme in the cathecolaminergic pathway, is absent in dopaminergic neurons [Zetterström et al., 1997]. On the other hand, mice lacking the D2 receptors for dopamine show increase Nurr1 expression in mesencephalic dopaminergic neurons, suggesting that actions mediated by D2 receptors might be a consequence of altered expression of Nurr1 [Tseng et al., 2000]. In addition, Nurr1 enhances the transcription of the human dopamine transporter gene [Sacchetti et al., 2001], one of the most specific phenotypic markers for dopaminergic neurons, and of the tyrosine hydroxylase gene [Sakurada et al., 1999; Kim et al., 2001, 2003]. Studies in Nurr1-null heterozygous mice show that Nurr1 increases spontaneous locomotor activity in response to stress [Eells et al., 2002]. The effect of amphetamines in Nurr1-null heterozygous locomotion remains controversial [Eells et al., 2002; Bäckman et al., 2003].

These observations suggest that NR4A2 by itself, or through heterodimerization partners, may participate in diseases with altered dopaminergic function such as Parkinson's,

schizophrenia, and drug abuse. NR4A2 may also be implicated in some personality traits with increased vulnerability to stress [Eells et al., 2002] such as those in the schizophrenia phenotype spectrum. Both mutations associated with decreased or increased NR4A2 activity and with altered regulation of the *NR4A2* gene throughout development might be associated with disorders such as schizophrenia and Parkinson's. Future studies must address whether the expression of NR4A2 is altered in the brain of schizophrenic patients or influences their response to alcohol exposure or drug treatment. Better understanding of the pathways involving NR4A2 might make it a potential target for therapy with drugs like 6-mercaptopurine analogs or even stem-cell transplants as recently suggested [Ordentlich et al., 2003].

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