Evaluation of the Role of Nurrl in a Large Sample of Familial Parkinson’s Disease

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Abstract: Parkinson’s disease (PD) is a common neurodegenerative disorder in humans with wide variability in the age of disease onset. Although the disease has been thought previously to occur sporadically in most patients, there is increasing evidence of a genetic contribution to the disorder. Recently, a polymorphic variant within intron 6 of the Nurrl gene was reported to be associated with sporadic and familial PD. In an effort to identify susceptibility genes for PD, we have collected 783 PD patients from 372 families and 397 healthy controls from 217 families. PD patients and healthy controls were genotyped for the intron 6 insertion polymorphism by restriction endonuclease digestion. No significant difference in either homozygosity or heterozygosity for the 704G7049 (IVS6 1361 +166G) polymorphism was detected in the PD patient cohort as compared with the panel of healthy controls. Moreover, direct sequencing of exon 1 of the Nurrl gene in PD patients failed to detect either of the two recently reported Nurrl mutations identified in a small subset of a PD patient cohort. Taken together, these data suggest that genetic alteration at the Nurrl locus is not a significant risk factor for the development of Parkinson’s disease in our large sample of familial PD patients. © 2004 Movement Disorder Society

Keywords: Nurrl; intron 6 polymorphism; familial Parkinson’s disease; susceptibility locus; genetic factors

Parkinson’s disease (PD) is the second most common neurodegenerative disorder affecting more than 1% of 55-year-old individuals and more than 3% of those over 75 years of age.1 The pathological features of PD include loss of dopaminergic neurons in the substantia nigra and the presence of intracytoplasmic inclusions, called Lewy bodies, in nigral and extranigral neurons.2-4

Recent studies suggest that genetic factors play an important role in determining the pathogenesis of PD, with the risk of PD being from 2 to 14 times higher for first degree relatives of an affected individual as compared with members of unaffected families.5-15 Mutations in four genes have been found in families segregating autosomal dominant or autosomal recessive PD: α-synuclein (PARK1,14 PARK415), parkin (PARK2)16; ubiquitin carboxy-terminal hydrolase L1 gene (UCH-L1; PARK5)17; and DJ-1 (PARK7).18 Genetic analyses have detected linkage to several other chromosomal regions, although the genes have not yet been identified (PARK3,19 PARK6,20 PARK821).

Several recent reports have suggested that nucleotide substitutions at the Nurrl locus may play a role in the risk of developing PD.22-24 Also known as NR4A2, Nurrl encodes a member of a nuclear receptor superfAMILY that is essential for differentiation of nigral dopaminergic neurons.25-28 Mice in which both alleles of the
Nurr1 gene have been inactivated lack mesencephalic dopaminergic neurons. Mice in which only one copy of the Nurr1 gene is inactivated demonstrate greater susceptibility to nigral injury and have features consistent with PD. Taken together, these data suggest that Nurr1 may be a potential susceptibility gene for PD. Although a prior study of 20 Swedish PD patients found no significant difference in the frequency of a Nurr1 intron 6 polymorphism as compared with normal controls, Xu and colleagues reported that heterozygosity for what is commonly known as the 7048G7049 polymorphism in intron 6 (IVS6 136I +16insG, insertion of a guanine nucleotide between nucleotides 16 and 17 of intron 6, GenBank accession number NM_173172) was significantly more common among familial PD patients and sporadic PD patients as compared with healthy controls. More recently, an additional study in an independent sample suggests that heterozygosity for this polymorphism confers an increased risk for PD. In addition to the intron 6 polymorphism, two mutations in the 5′ untranslated region (exon 1) of the Nurr1 gene have been recently reported in a subset of familial PD subjects. In an effort to identify susceptibility genes for PD, we have collected DNA samples from 783 PD patients from 372 families. We now report on analysis for the intron 6 polymorphism of the Nurr1 gene using the largest sample of patients with PD to date.

SUBJECTS AND METHODS

Subjects

Families consisting of at least one pair of living siblings diagnosed with PD were recruited through 59 Parkinson Study Group (PSG) sites located throughout North America. Informed consent was obtained from all study participants (n = 783). A family history questionnaire was completed for each pedigree that identified all family members diagnosed with PD or who were believed to be showing signs of PD. All study participants completed a uniform clinical evaluation (Unified Parkinson’s Disease Rating Scale [UPDRS] Parts II and III) and a diagnostic checklist. The checklist was developed so as to provide inclusion criteria consisting of clinical features highly associated with autopsy-confirmed PD and exclusion criteria highly associated with other non-PD pathological diagnoses. Responses on the diagnostic checklist were used to classify study subjects as having verified PD or nonverified PD.

Autopsies have been completed and a report generated for 7 study participants. These included 3 individuals who, based on their clinical evaluation, had been classified as verified PD, with an autopsy confirming this diagnosis. In addition, 3 of 4 individuals classified as nonverified PD also had pathology consistent with PD. This suggests that our classification of verified PD is quite conservative and provides further rationale for using a broad disease definition for genetic analyses.

Age of onset of PD was also collected for all affected study participants. In a subset of this sample (n = 149), it was shown previously that age of onset collected through medical record review, a written instrument, or queried orally for a case report form all provide highly reliable age of onset information. For the current analyses, therefore, the self-reported age of onset of PD recorded on the Study Visit case report forms was used for most study subjects.

A control sample of 397 healthy individuals from 217 twin families was ascertained as part of an ongoing study to identify genes contributing to healthy aging. These subjects were all male twins, members of the National Academy of Sciences-National Research Council (NAS-NRC) twin registry, and World War II veterans born between 1917 and 1927. They completed a health screen in 1998 and at that time had not been diagnosed with PD.

Molecular Analysis

Intron 6 7048G7049 Insertion Polymorphism

Total genomic DNA prepared from peripheral blood leukocytes obtained from the PD patients and healthy control individuals was PCR amplified for a 555/556-bp base fragment of the Nurr1 gene containing intron 6 using primers 5′-AAGATTCCTGCCTCGAGA-3′ and 5′-CCACCGAAGCATCAGCAACCT-3′. PCR products were digested with the restriction endonuclease BstRI and electrophoresed through 4% composite agarose to determine genotypes for the intron 6 7048G7049 insertion polymorphism (IVS6 1361 +16insG). DNA fragments were visualized by UV light after ethidium bromide staining. The IVS6 1361 +16insG polymorphism results in loss of a BstRI endonuclease site.

Exon 1 Mutation Detection

A 372-base pair fragment containing exon 1 of the Nurr1 gene was PCR amplified using primers 5′-CAGCG-GAGACTTTAAGTGTCAT-3′ and 5′-AGAGAACCCT- GTCCCCACACAA-3′ and genomic DNA from familial PD patients as well as the normal, healthy control individuals. The resulting PCR products for the PD patients and a subset of the controls were purified using the QIAquick 96 PCR purification kit (Qiagen, Santa Clara, CA) and sequenced on both strands using an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City,
**Statistical Analysis**

Statistical analyses were designed to test the primary hypothesis of whether the previously reported Nurr1 intron 6 insertion polymorphism was associated with PD. Fisher’s exact test was utilized to test whether the proportion of individuals homozygous for the IVS6 1361+16insG genotype was increased among those individuals with PD as compared with the controls. The Statistical Analysis System (SAS v.8.2; SAS Institute, Cary, NC) was employed for all analyses. Due to the collection of data from multiple individuals per family, 10,000 datasets were created by randomly sampling 1 individual from each PD and control family. The $\chi^2$ statistic was calculated for each of the 10,000 datasets. Common resampling techniques (bootstrapping) on the 10,000 $\chi^2$ statistics were employed to obtain a representative $\chi^2$ value. The median $\chi^2$ statistic from the resampling distribution and its corresponding $P$ value are reported.

Secondary analyses were carried out to test whether the Nurr1 polymorphism confers greater PD susceptibility in a subset of the sample. Additional analyses were carried out utilizing only those families having at least 1 member with an age of PD onset of 45 years or earlier ($n = 57$ families). Due to the presence of families with parkin mutations, particularly among the early-onset sample, the role of the intron 6 polymorphism was also examined separately in the sample of early-onset families without parkin mutations ($n = 36$). The families with later onset PD (age of onset >45 years in all affected individuals) were analyzed separately ($n = 306$ families). Linkage to chromosome 2q had been reported previously in our kindreds with a strong family history of PD. A strong family history was defined as 4 or more affected individuals in the same family or an affected sibling pair who also had a parent with PD. The frequency of the IVS6 1361+16insG homozygous genotype in subjects having a strong family history of PD ($n = 110$ families) therefore was compared with that observed in the healthy control subjects. Those families for which all affected individuals were classified as verified PD ($n = 199$) and those families classified as Caucasian ($n = 342$) were also analyzed separately to determine an increased susceptibility to PD based on Nurr1 genotype in either of these subsets.

**RESULTS**

A total of 372 families consisting of at least 1 pair of living siblings diagnosed with PD were recruited through...

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FIG. 1. BstEII restriction endonuclease digestion of Nurr1 intron 6 PCR products. Insertion of a G nucleotide between nucleotides 7048 and 7049 results in loss of a BstEII restriction endonuclease site. Individuals homozygous for the more common allele demonstrate complete digestion of the 555-base pair (bp) PCR product into products of 434 and 121 bp [lanes 4–6, 10, 16] whereas individuals homozygous for the G insertion demonstrate only the 555-bp product [lanes 10, 16]. Individuals heterozygous for the G insertion have all three products [lanes 1–3, 7–9, 11, 12,14]. Asterisk, position of G insertion resulting in loss of the BstEII site.

59 Parkinson Study Group (PSG) sites located throughout North America. Most individuals with PD (736/783, 94%) were Caucasian; 37 subjects were Hispanic (4.7%) and 10 subjects (1.3%) were Asian, Pacific Islander, or African American. The average age of onset of the PD subjects was 60.9 years (age range, 18–87 years). The 397 healthy, control twins consisted of Caucasian males with an average age of 72.4 years (age range, 71–82 years) at the time of their most recent questionnaire evaluation when they indicated that they did not have a diagnosis of PD.

In total, 742 PD patients and 397 normal, healthy controls were genotyped successfully for the intron 6 insertion polymorphism. As shown in Figure 1, the insertion polymorphism results in loss of a BstEII endonuclease site. In our sample, the average frequency of the homozygous IVS6 1361 +16insG polymorphism in the PD families was not significantly increased ($P = 0.32$) from that observed in our healthy control subjects (Table 1). Heterozygosity for the polymorphism was likewise very similar between PD patients and controls ($P = 0.20$). The frequency of the homozygous IVS6 1361 +16insG genotype in those families having at least 1 member with early onset PD ($n = 57$) was not significantly different from that observed in the healthy controls ($P = 0.56$). When the 36 early-onset families without a parkin mutation were analyzed separately, the frequency of individuals homozygous for the IVS6 1361 +16insG was not increased significantly over the frequency found among controls ($P = 0.37$). Comparison of the frequency of the homozygous genotype in the fami-
lies with later onset PD (n = 306) was also not statistically different from that found in controls (P = 0.47).

Comparison of the frequency of the homozygous polymorphism in a sample of individuals having a stronger family history of PD (n = 110) was also not significantly greater than that in the control sample.

Because all control subjects used in this study were Caucasian, an analysis was carried out using only those PD families that were Caucasian (n = 342). The frequency of PD patients homozygous for the intron 6 insertion polymorphism (4.7%) was not significantly different from that observed for control subjects (3.9%, P = 0.49). Finally, stratification was carried out based on disease classification and the analysis utilized only those 199 families for which a PD patient was classified as having verified PD based on the administered diagnostic checklist. The frequency of homozygosity for the insertion polymorphism was 4.4% in this subset, which is not significantly different from the frequency in the controls (P = 0.54).

Direct sequencing of exon 1 of the Nurr1 gene was also carried out in the sample of PD patients (n = 774) and in 46 unrelated, healthy control subjects (92 normal alleles) to assess the frequency of the −291Tdel and −245T→G mutations recently reported by Le and colleagues. The authors reported on these two mutations with a combined frequency of ~5% in a sample of 107 individuals with familial PD. Our direct sequence analysis of 774 familial PD patients failed to detect either of the exon 1 mutations reported by Le and associates. Sequencing of 1,636 exon 1 alleles of the Nurr1 gene in our familial PD sample (1,548 alleles) and 46 unrelated, healthy control subjects (92 alleles) yielded only a single heterozygous change in one of the familial PD patients: −145G→C. Interestingly, this single nucleotide change was based on comparison of the obtained sequences to transcript variant 3 of the Nurr1 cDNA (GenBank accession number NM_173173). Examination of the three other Nurr1 cDNA variants (GenBank accession numbers NM_173173, NM_173171, NM_006186), however, as well as a chromosome 2 genomic contig containing the sequence of the Nurr1 gene (NT_005403) identifies this as a potential polymorphism with two of four cDNA sequences and the genomic sequence having a C in place of the G. No novel sequence variants were thus identified in exon 1 of the Nurr1 gene in our sample of 774 PD patients.

DISCUSSION

As part of an ongoing study to determine the contribution of genetic factors to the pathogenesis of PD, we recruited 372 families consisting of at least one parent of living siblings diagnosed with PD through 59 PGs sites located throughout North America. Carrying out a genome screen to identify PD susceptibility genes in those individuals determined not to carry a parkin mutation, we have found strong evidence for linkage to chromosome 2 and chromosome X. When only those families with the strongest family history of PD (4 or more affected individuals or an affected sibling pair with an affected parent) were analyzed, significant evidence of linkage to chromosome 2q36–37 was obtained. Recently, the Nurr1 gene, located on chromosome 2q, has been implicated in both mouse and human studies as a potential candidate gene for PD susceptibility.

Although the Nurr1 gene is located more than 50 centimorgans away from our previously detected region of linkage on chromosome 2q36–37 and we have no evidence for linkage to the Nurr1 locus (LOD = 0.4), we nonetheless felt it important to test whether variation in the Nurr1 gene might contribute to PD susceptibility in our sample. Based on previous reports, we tested the intron 6 insertion polymorphism (IVS6_1361 +16insG) to determine whether homozygosity or heterozygosity of the insertion polymorphism is at higher frequency in a familial PD sample as compared with a sample of healthy controls. In addition, because mutations in exon 1 of the Nurr1 gene were reported to cause PD in some
familial PD samples,22 we also sequenced exon 1 in our multiplex PD patients as well as a subset of our healthy controls.

Our sample of 783 PD patients represents the largest study to date of Nurr1 and PD. Our analysis for the intron 6 insertion polymorphism found no significant differences in the frequency of the IVS6 1361 +16insG allele or the three possible genotypes as compared with a panel of normal, healthy controls. Our results are in contrast to two recent reports examining the relationship of genotype at the intron 6 insertion polymorphism and the incidence of PD. Xu and colleagues23 studied 105 patients with familial PD and 120 patients with sporadic PD, and genotypes at the intron 6 polymorphism were determined and compared with those of 221 age-matched healthy controls. Although only 0.9% of controls were homozygous for the polymorphism, 9.5% of familial PD patients and 4.2% of sporadic PD patients were determined to be homozygous. The most striking difference between our study and that of Xu and coworkers23 is the frequency of control individuals homozygous for the intron 6 insertion polymorphism: 3.9% in our study and only 0.9% in that of Xu and associates.22

Zheng and colleagues (2003)24 recently reported an increased frequency of heterozygosity for the intron 6 polymorphism among 103 PD patients as compared with a sample of 88 controls. Importantly, the frequency of intron 6 insertion polymorphism homozygotes in their study (4.9% in PD patients and 4.5% in controls) is relatively similar to the rate observed in our sample. Unlike Zheng and coworkers,24 we did not find a significant increase in heterozygosity for the intron 6 polymorphism in our familial PD sample. In our sample, the frequency of PD heterozygotes was 32.9% among all patients, varying from 32.7% to 38.2% with stratification by age of onset, disease classification, ethnicity, or strong family history of PD. In the previous study by Zheng and associates,24 the frequency of heterozygotes varied between 23.8% and 55.6% with stratification and an overall frequency of 39.8% among all patients. Stratifying our sample by age of onset did not alter significantly the frequency of heterozygotes in our PD sample whereas Zheng and colleagues24 found a significant increase in heterozygotes among early-onset (≤45 years) versus late-onset PD.

The increased frequency of heterozygotes reported by Zheng and coworkers24 among early-onset cases used spousal controls as a comparison group. The frequency of heterozygotes among control subjects was higher in the current study than in the study of Xu and associates (20.3%)22 or Zheng and colleagues (25.0% overall, and 27.6% among the subset of spousal controls),24 raising the possibility that the control subjects in the current study are not representative of the population from which the PD patients were derived. In our study, the control subjects were all Caucasian male twins, National Academy of Sciences–National Research Council (NAS-NRC), World War II veterans born between 1917 and 1927.28 To preclude possible ethnic differences in the frequency of the two intron 6 alleles, we carried out a secondary analysis comparing the Caucasian controls to the 342 Caucasian PD families. The frequency of heterozygosity for the intron 6 insertion polymorphism in the Caucasian PD sample (32.8%) was not significantly different from that in the control group, which consisted of only Caucasians (37.4%, P = 0.20).

The question of whether the control subjects in our study are representative of our PD patient population cannot fully account for the discrepancies between these studies. For example, the relatively high frequency of heterozygotes among early-onset PD patients reported by Zheng and colleagues (55.6%)24 was not reproduced among the early-onset PD cases of the current study (34.2%). Furthermore, previously reported associations with the homozygous and the heterozygous intron 6 polymorphism each were found in only one of the two prior studies,22,24 and neither was confirmed in the current study.

Using a variety of different statistical and molecular methods, we did not find any evidence that Nurr1 is an important susceptibility locus in our familial PD sample. We found no evidence of linkage to the chromosomal region harboring Nurr1. There was also no association between the intron 6 polymorphism and disease risk. Consistent with these results, we failed to detect any mutations in exon 1 of the Nurr1 gene in our PD patients. We thus believe Nurr1 is unlikely to be a major susceptibility locus for familial PD.

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APPENDIX

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