Functional association of the parkin gene promoter with idiopathic Parkinson’s disease

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INTRODUCTION

Parkinson’s disease (PD; MIM 168600) is the second most common neurodegenerative disorder, presently affecting more than a million people in the USA. Clinically, the disease is characterized by symptoms including resting tremor, brady-kinesia and rigidity, due to the specific loss of pigmented dopaminergic neurons in the substantia nigra (1). Recent evidence has suggested that genetic components may be important in familial early-onset parkinsonism as well as in more typical, idiopathic PD (2–6).

Deletions/duplications and point mutations in the parkin gene were originally associated with autosomal recessive juvenile parkinsonism (AR-JP), without Lewy body pathology (7). Compound heterozygous mutations have since been identified in cases resembling typical late-onset PD (2,8), and in a single case with Lewy bodies observed on postmortem examination (9). In addition, apparently dominant families have been described with only a single parkin mutation in affected cases (9–14).

Subsequently, several studies have hypothesized that loss of parkin expression due to haploinsufficiency may be a risk factor for dopaminergic cell death. Of note, in one family, [¹⁸F]fluorodopa positron emission analysis of parkin mutation carriers was also suggestive of dopaminergic loss compatible with presymptomatic disease (10,14).

With this background, we hypothesized that reduced rather than completely ablated parkin gene function could confer risk for idiopathic PD. Polymorphic variability within a gene promoter can effect gene expression and has previously been associated with age-associated neurodegeneration (15–17). Recently, nine single-nucleotide polymorphisms (SNPs) have been described in the parkin gene promoter (11). Two of these SNPs reside within the parkin core promoter, i.e. the minimum region of DNA upstream of parkin exon 1 that contains full transcription activation in neuroblastoma cells. In the present study, we describe a genetic association of idiopathic PD with the −258 T/G promoter SNP in a large, clinically well-characterized case/control series. Further, we demonstrate this SNP occurs within a nuclear protein-binding site and affects transcription.

RESULTS

We previously described nine SNPs in the parkin gene promoter and their heterogeneity in Northern Europeans (11). Two of the nine polymorphisms (−258 T/G and −227 A/G) lie within the parkin core promoter, i.e. the minimum region of DNA upstream of parkin exon 1 that contains full transcription activation in neuroblastoma cells. These SNPs were genotyped in a population-based series of PD cases and controls; both were in Hardy–Weinberg equilibrium (not shown). Genotype distributions of the −227 A/G SNP were not significantly different between PD cases and controls. However, the −258 SNP, specifically the −258 G allele, demonstrated some evi-
of the protein–probe interaction is suggested by the reduction of the shifted complex upon addition of unlabeled probe. Both the T and G allele-specific probes completely competed away the shifted complex at a 40-molar excess to labeled T allele probe. However, at lower concentrations of competitor probe, the G allele did not compete the shifted complex as efficiently as the T allele, suggesting that the −258 T-to-G alteration may reduce nuclear protein-binding affinity. This is illustrated in Fig. 2, as a 5-fold excess of the T allele competes away the complex similarly to a 40-fold excess of the G allele. Results were typical of multiple experiments.

To determine the in vivo effects of the −258 T/G allele on transcription regulation, we cloned allele-specific promoter fragments (Fig. 1) into the pGL3-Basic vector (Promega). A knockout promoter fragment was also designed with multiple mutations across the consensus TTGGC NF1-A1-binding motif (Fig. 3), which have been previously shown to negate interactions with nuclear protein (21). Promoter activity was assayed using a dual-luciferase system (Promega), and compared relative to SV40 control promoter activity. Of note, the −258 G allele reduced luciferase activity by ~25% relative to the −258 T allele. The NF1-A1 knockout vector also reduced luciferase activity by ~25%, illustrating the importance of the −258 nucleotide regarding transcription regulation (Fig. 3). Allele-specific constructs were also designed for the −227 A/G SNP; however, no differences in luciferase activity were observed (data not shown).

**DISCUSSION**

Recessive and compound heterozygous mutations in the parkin gene clearly cause early-onset parkinsonism. However, the relationship of parkin variability to idiopathic, Lewy body PD remains obscure. Initial studies of parkin coding polymorphisms suggested an association with PD (23–25), but these early studies have been difficult to reproduce (26,27). There are a number of possible confounders, including clinical, locus and allelic heterogeneity in the populations under study (28). Criteria to improve replication validity have been proposed (29);
however, functional analysis of allelic variability might demonstrate a direct effect on gene/protein expression.

The position of the parkin promoter and SNPs within this region have been previously described (11,18). We evaluated two of these SNPs based on their location in the parkin core promoter for association with idiopathic PD in a clinically well-defined series of idiopathic PD cases versus controls. While the −227 SNP showed no difference between cases and controls, the −258 G allele was subsequently found to be over-represented in PD cases (>71 years). In silico analysis also predicted the SNP at −258 to affect protein binding, while the SNP at −227 was not predicted to be near a known protein-binding domain.

Gel-shift experiments (EMSA) verified that sequence about the −258 nucleotide binds nuclear protein derived from human substantia nigra. Whether the −258 NF1-A1 consensus site is occupied in the parkin promoter in vivo remains to be determined (specific antibodies to this protein were not available for competition experiments). However, dual-luciferase measurements confirmed that −258 T/G allelic differences affect parkin gene expression in transient transfection assays.

This study provides the first functional evidence implicating parkin promoter SNP variability in gene expression. Furthermore, the data support a genetic association with late-onset Parkinson’s disease. The −258 G allele was observed in 19% of controls and 25% of late-onset PD cases (>71 years). These findings are consistent with a previous study of in which the −258 G allele was observed in 14% of Northern European controls and in 61% (11/18) of alleles inherited by heterozygous parkin carriers (OR = 9.7, 95% CI = 2.8 – 33.9, P < 0.001) (11). When inherited in trans, the −258 G allele associated with reduced parkin expression may also contribute to early-onset parkinsonism in carriers with a parkin mutation.

MATERIALS AND METHODS

Patients with Parkinson’s disease and controls

Cases with PD and controls were derived from an ongoing study of the epidemiology and genetics of PD at Mayo Clinic Rochester (30,31). A total of 319 unrelated PD patients and 196 controls who provided blood samples were included in this study. The majority of PD patients (n = 278; 87%) were sequential new referrals to the Department of Neurology, between July 1996 and May 1999, and were all residents of Minnesota or the surrounding four states (Wisconsin, Iowa, South Dakota and North Dakota). Forty-one additional patients (13%) were recruited through an incidence study of PD in the Olmsted County, Minnesota, population. Controls were either spouses of patients free of PD at the time of the study (n = 150; 77%) or subjects free of PD from the same population (n = 46; 23%).

All subjects were examined using a standardized clinical protocol by one of three movement disorders specialists (Drs D. M. Maraganore, J. E. Ahlskog and J. H. Bower of the Mayo Clinic) and had at least two of four cardinal signs (bradykinesia, rigidity, rest tremor and postural instability). Subjects with no or minimal improvement from levodopa (in combination with carbidopa) at doses >1 g/day were excluded. We also excluded patients with other causes of parkinsonism, or with unexplained signs of more extensive neurological involvement (dementia or mild dysautonomia were allowed if they occurred after the first year of motor symptoms).

This study was approved by the Mayo Institutional Review Board, and informed consent was obtained from each subject at the time of blood drawing. All laboratory tests were performed by an investigator kept unaware of the case–control assignment of subjects. Blood samples were processed via the Purgene procedure (Gentra Systems, Inc., Minneapolis, MN) to extract DNA.

Genetic analysis

SNPs were determined using a standard RFLP protocol, first amplifying 25 ng of patient genomic DNA (primers: forward 5’-GCAITTTGTTAAGCTCCAGGGTCTC-3’ and reverse 5’-CCTGCTGGAGGTGCAGTTCTC-3’ for the −258 and −227 variants) using a 60–50°C touchdown protocol over 35 cycles. PCR products were then digested with a restriction

Figure 1. A schematic representation of the parkin core promoter. The parkin exon 1 is shown as bold sequence, with the parkin start codon highlighted (double-underlined). The two SNPs in this region are indicated with stars and the DNA sequence used as an EMSA probe is boxed. The positions of the primers used to generate the luciferase constructs are overlaid.
enzyme (StuI for the \(\text{C}0\)227 variant and AlwNI for the \(\text{C}0\)258 variant; New England Biolabs) (Table 1). Digested products were analyzed on 3% agarose gels, stained with ethidium bromide.

**Statistical analysis**

The association of the candidate gene with PD was measured by ORs, which closely approximate the relative risk in rare disease. Because more men were included among cases and more women among controls, ORs were adjusted for sex using logistic regression models (32). Similarly, ORs were adjusted for age at examination (time of the study) where appropriate. For each OR, a 95% CI was computed, and a two-sided statistical test was performed at an \(a\)-level of 0.05. Analyses restricted to subjects with both parents of European origin were conducted to reduce the risk of population stratification bias. Analyses stratified by age at examination, gender (not shown) and family history (not shown) were also performed. All analyses were performed using SAS software (Cary, NC).

**Electromobility shift assays (EMSA)**

Nuclear protein was derived from human fresh-frozen substantia nigra tissue (courtesy of Dr Deborah Mash and the University of Miami/National Parkinson’s Foundation Brain Endowment Bank) using the Sigma Nu-CLEAR kit (Sigma Life Sciences), according to the suggested protocol. Probes to detect the \(-258\) SNP were made by Invitrogen (forward 5’-GGCAGGACCTTGCTAGAGCTG-3’ and Reverse 5’-CAGCTCTAGCCAGGTCTCTGCC-3’ for the \(-258\) T variant, and forward 5’-GGCAGGACCTGCTTAGAGCTG-3’ and reverse 5’-CAGCTCTAGCCAGGTCTCTGCC-3’ for the \(-258\) G variant) and cartridge-purified to select for full-length oligonucleotides. The two \(-258\) variant-specific double-stranded oligonucleotides were generated by heating the complementary oligonucleotides in a high-salt solution (10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) at 65°C for 15 min, and then allowed to cool to room temperature. Double-stranded DNAs were labeled using \([\gamma-^{32}\text{P}]\text{dATP (3000 mCi/mmol, NEN)}\) and T4 polynucleotide kinase (Promega), and radioactivity was counted by liquid scintillation. The Gel-Shift Assay system (Promega) was employed, following the manufacturer’s protocol, and allele-specific competition reactions were carried out in tandem. Products were electrophoresed in Novex 6% DNA retardation gels, in 0.5× TBE running buffer at 100 V, and gels were dried and visualized using Kodak Biomax film with one intensifier screen at \(-70^\circ\text{C}\) overnight.

**Construction of luciferase vectors**

Three parkin core promoter constructs, containing the \(-258\) T allele, the \(-258\) G allele or an NF1-A1 consensus site knockout (21), were amplified from BAC DNA containing parkin exon 1, using primers with internal restriction sites for cloning (forward 5’-GGAAGAGGTACCGACCTTGGCTAGCTGTA-3’ for the \(-258\) T allele, forward 5’-GGAAGAGGTACCGACCTGGCTAGCTGTA-3’ for the \(-258\) G allele, forward 5’-GGAAGAGGTACCGACCTGTA-3’ for the NF1-A1 knockout mutant and reverse 5’-CGGTGTTGACCGTCGCTAGCCA-3’)(Fig. 1). PCR was performed using a 65–55°C touchdown protocol, with Taq DNA polymerase (Qiagen) and 1 ng of BAC DNA. PCR products and the pGL3-Basic vector (Promega) were digested with KpnI and Nhel (Roche Biochemicals) and purified (Qiagen), according to the manufacturers’ conditions. Vector arms were
dephosphorylated (CIP; Promega) and ligated to digested PCR fragments (DNA Rapid Ligation Kit; Roche Biochemicals). Constructs were subcloned into DH5α cells (Life Technologies), single colonies were miniprepped (Qiagen) and the insert was verified by sequence analysis.

Plasmid transfection and luciferase assay

Human dopaminergic neuroblastoma BE(2)-M17 cells and human embryonic kidney cells (HEK-293T) were cultured in Opti-MEM (Life Technologies) supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were plated 24 h prior to transfection into 24-well culture plates at 80% confluence and maintained in an atmosphere of 5% CO₂ at 37°C. Transfection was performed with Fugene (Roche Biochemicals), using 0.2 μg of DNA per well, in a 1:3 ratio of DNA : Fugene reagent, and added to cells in serum-free media for 12 h.

Luciferase-containing constructs (pGL3) were co-transfected with phRL-TK synthetic renilla vector (Promega) to control for transfection efficiency, in a molar ratio of 1:100 (phRL-TK versus pGL3). Forty hours after transfection, cells were gently rinsed with PBS and then harvested with Passive Lysis buffer (Promega). The Dual Luciferase system (Promega) was used according to the manufacturer’s protocol, and experiments were repeated in six independent wells. Readings were taken in duplicate on a Turner Designs 20/20 Single Injector Luminometer.

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