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Association of polymorphisms and reduced expression levels of the *NR4A2* gene with Parkinson's disease in a Mexican population

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ABSTRACT

Introduction: The NR4A2 transcription factor is important in the development, survival and phenotype of dopaminergic neurons and it is postulated as a possible biomarker for Parkinson's disease (PD). Therefore, our aim was to analyze in a sample of a Mexican population with idiopathic PD, mutations (in two hotspot mutation regions) and two polymorphisms (rs34884856 in promotor and rs35479735 intronic regions) of the *NR4A2* gene. We also evaluate the levels of *NR4A2* gene expression in peripheral blood for a Mexican population, and identify whether they are associated with *NR4A2* gene polymorphisms.
Methods: We conducted a case-control study, which included 227 idiopathic PD cases and 454 unrelated controls. Genetic variants of the *NR4A2* gene were genotyped by high-resolution melting (HRM) and validated by an automated sequencing method. The gene expression was performed in peripheral blood using a real-time polymerase chain reaction.
Results: The rs35479735 polymorphism was associated with a higher risk of developing PD. In addition, *NR4A2* gene expression was significantly decreased in patients with PD. Linkage disequilibrium analysis showed a haplotype H4 (3C-3G) that showed lower levels of expression, and contained the risk alleles for both polymorphisms.
Conclusions: In summary, this is the first study in a Mexican population that considers the analysis of *NR4A2* in patients with PD. An association was identified between genotype and mRNA expression levels of *NR4A2* in patients with PD. These results suggest that polymorphisms and expression of the *NR4A2* gene could play an important role in the risk of developing PD in Mexican populations.

1. Introduction

Parkinson's disease (PD) is characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain and subsequent dopamine depletion in the striatum [1]. Identification of genes controlling the maintenance of dopaminergic neurons, such as NR4A2 (also known as Nurr1), have provided new insight into the mechanisms leading to neuronal death in PD, which so far is not well understood.

The NR4A2 transcription factor is important in the development, survival and phenotype of dopaminergic neurons (for a review see Decressac et al., 2013 [2]). This orphan nuclear receptor is encoded by the *NR4A2* gene that is located on chromosome 2q22-23 and consists of eight exons [3]. It is an immediate early gene, and highly expressed

Abbreviations: ACD, Acid citrate dextrose; AIC, Akaike information criteria; AIM, ancestry-informative markers; ASM, automated sequencing method; CI, confidence interval; H, Haplotype; HWE, Hardy-Weinberg equilibrium; HRM, High-Resolution Melting; Indel, Insertion or deletion of bases in the DNA; LD, Linked disequilibrium; NR4A2, Nuclear Receptor Subfamily 4 Group A Member 2; OR, odd ratio; PD, Parkinson's disease; PBMC, Peripheral blood mononuclear cells; PCR, Polymerase chain reaction; SNP, Single nucleotide polymorphism; UPDRS, Unified Parkinson's Disease Rating Scale; 3G, Insertion G; 2G, Deletion G; 3C, Insertion C; 2C, Deletion C.

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in dopaminergic neurons [2] and other cells, including peripheral blood leukocytes [4].

Mutations in the *NR4A2* gene are associated with familial PD and are mainly identified in exon 1 and 3. These mutations are linked with a significant decrease in *N4R42* mRNA [5,6]. Furthermore, different polymorphisms of *NR4A2* are described in diverse populations, for example, rs34884856 in the promoter and rs35479735 in the intron 6 regions are polymorphisms present in Caucasian and Asian populations but with different frequencies [7–10]. In particular, the rs35479735 polymorphism (insertion/deletion of a G) in *NR4A2* is associated with familial and sporadic PD in Caucasian and Asian populations [11–13]. In addition, rs34884856 polymorphism (insertion/deletion of a C) has been linked with disease related to the dopamine system [14,15].

In various studies, *NR4A2* gene expression in peripheral blood has been suggested as a possible biomarker for sporadic PD. In these studies, *NR4A2* gene expression was reduced in human peripheral blood mononuclear cells (PBMC) of Caucasian and Asian patients with sporadic PD [16–18]. This is consistent with studies of PBMC in PD patients that showed changes in gene expression that may be related with neurodegeneration [19,20]. Therefore, gene expression of *NR4A2* could help in the early diagnosis of the disease and in the evaluation its progression from a clinical perspective.

Because of the relationship of NR4A2 in the dopamine system and its association with PD, our aim was to analyze in a sample of a Mexican population with idiopathic PD, mutations (in two regions with a high mutation rate) and two polymorphisms of the *NR4A2* gene (rs34884856 and rs35479735). In addition, the levels of *NR4A2* gene expression were analyzed in PBMC for a Mexican population. We also evaluated the possible association between levels of NR4A2 gene expression and the polymorphisms analyzed for this gene.

2. Materials and methods

2.1. Participants

A total 227 sporadic PD cases and 454 unrelated controls were examined. Patients with idiopathic PD from the movement disorder clinic at the National Institute of Neurology and Neurosurgery of Mexico were included. A movement disorder specialist examined these patients, all of whom fulfilled the UK PD Society Brain Bank Clinical Diagnostic Criteria of PD [21]. Control subjects were recruited under the following inclusion criteria: without a diagnosis of neurodegenerative disease, with no family history of movement disorders and without severe, uncontrolled physical disease (e.g., cancer, hypertension and diabetes). The institutional ethics committee approved this study and all the patients gave their informed written consent. Our study was submitted according to the principles of the Helsinki Declaration. Patients and controls were Mexican mestizo, that is, subjects who were born in Mexico, had a Spanish-derived last name and a family of Mexican ancestry that could be traced back to the third generation [22]. To restrict the possible confounding effects of age and gender, frequency and individual matchings were carried out, and a ratio of 1 case per 2 controls was applied. Whole blood was collected in 6 ml Acid Citrate Dextrose (ACD) tubes from patients and controls for genotyping analysis and measurement of gene expression levels of *NR4A2*.

2.2. Genotyping of NR4A2

Genomic DNA was extracted from PBMC by standard procedures [23]. Mutations and polymorphisms (Indel) were selected from the promoter, exon and intronic regions of the *NR4A2* gene using the Single Nucleotide Polymorphism (SNP) database, and referenced sequences were taken from the dbSNP public database [Website 1]. Mutations were scanned in two region-high mutation rates (hotspot mutations).

Genetics variants were studied using high-resolution melting (HRM), and 20% of samples taken at random were sequenced as a quality control using an ABI3730 sequencing equipment (Applied Biosystems, Carlsbad CA, USA), and were genotyped with a 99% concordance for validation. Polymerase chain reaction (PCR) amplification for automated sequencing and the HRM for analysis of the genetics variants was performed using the Rotor-Gene 6000 instrument (Corbett Research Pty Ltd., Sydney, Australia). The primers used are listed in Table S1.

2.3. Quantitative reverse transcription PCR (qRT-PCR)

We evaluated *NR4A2* mRNA levels in the first 156 patients with PD and 102 control individuals. The gene expression was performed in PBMC from patients with PD and control participants using a method described previously [24]. Total RNA was extracted from PBCM using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. RNA integrity was determined by agarose gel electrophoresis and the concentration and purity were measured spectrophotometrically. The cDNA was generated from 1000 ng of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μl.

GAPDH was used as an internal reference gene to normalize target gene transcript levels. The quantitative PCR reactions were performed using predesigned primer and probes from Applied Biosystems (Foster City, CA, USA). The relative fold changes were determined by the method of $2^{-\Delta\Delta Ct}$ as described previously [25].

2.4. Statistical analysis

Demographic and clinical variables between PD and control groups were analyzed using SPSS software v.12.0 (SPSS Inc., Chicago, IL, USA) and STATA software v.11 (StataCorp, College station, Tx, USA). Numeric variables not normally distributed are presented as median and range and were compared using the Mann-Whitney *U* test. Comparison between categorical variables and deviations from Hardy-Weinberg equilibrium (HWE) were analyzed with chi-square tests, and are presented as absolute frequencies and proportions. The inheritance hypothesis was tested according to models: dominant, recessive, and heterozygous. Logistic regression analysis was used in a bivariate way to estimate the risk of polymorphisms with Parkinsons's disease. Multiple logistic models were constructed in order to identify the variables that explain better the risk of developing Parkinson's between the studied groups. Models were constructed including variables with biological relevance or with statistical significance, or both. Confounding bias was accepted when changes in estimated odds ratios (ORs) were equal or larger than 10%. When a principal effects model was reached, effect modification was also tested and interaction terms were constructed between the polymorphism, age and sex; the terms were included in the model when the significance of the *p*-value was larger or equal to 0.20. A Hosmer-Lemeshow Goodness of Fit test was performed for each multiple logistic model, based on Akaike information criteria (AIC). Pairwise linkage disequilibrium (LD, D´) estimations between polymorphisms and haplotype reconstruction were performed with Haploview version 4:1 [26] (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA). Statistical power to detect an association of rs34884856 and rs35479735 with PD was 0.28 and 0.93, respectively, as estimated with QUANTO software [Website 2].

Correlations between *NR4A2* gene expression levels, genotype and inheritance models were tested using Kruskal-Wallis or Mann-Whitney *U* tests. Statistical significance was set at $p \leq 0.05$.

3. Results

Demographic and clinical characteristics of PD patients and controls included in the study are shown in Table 1. Similar age and gender distribution were observed among individuals.