

## SHORT COMMUNICATION

# The screening of the 3'UTR sequence of *LRRK2* identified an association between the rs66737902 polymorphism and Parkinson's disease

Lucía F Cardo<sup>1</sup>, Eliecer Coto<sup>1,2</sup>, René Ribacoba<sup>3</sup>, Ignacio F Mata<sup>4,5</sup>, Germán Moris<sup>3</sup>, Manuel Menéndez<sup>3</sup> and Victoria Alvarez<sup>1</sup>

Mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) are the most common genetic determinants of familial and sporadic Parkinson's disease (PD). Most of the mutational screenings analyzed the exon-coding sequence. Our aim was to determine whether *LRRK2* 3' untranslated region (UTR) variants were associated with the risk of developing PD in a large cohort of patients ( $n=743$ ) and controls ( $n=523$ ) from Spain. We identified a total of 12 3'UTR variants (two new). Single-nucleotide polymorphism (SNP) rs66737902 C allele was overrepresented in patients ( $P=0.005$ ; odds ratio = 1.47). This SNP was in linkage disequilibrium with the p.R1441G mutation, but the association remained significant among mutation-negative cases. We found a significant lower level of the *LRRK2* transcript in the *Substantia nigra* (SN) of PD postmortem donors ( $n=9$ ) who were rs66737902 C carriers ( $P=0.01$ ). This SNP was predicted to affect a binding site for miR-138-2-3p. We showed that this microRNA was expressed in all the SN samples. In conclusion, we found a significant association between SNP rs66737902 and the risk of developing PD. This effect on PD risk could be explained by differences in *LRRK2* transcript levels between the two alleles.

*Journal of Human Genetics* (2014) 59, 346–348; doi:10.1038/jhg.2014.26; published online 24 April 2014

**Keywords:** DNA polymorphisms; genetic risk; *LRRK2* gene; microRNAs; Parkinson's disease

Parkinson's disease (PD) affects 1–2% of people older than 60 years.<sup>1</sup> In addition to rare mutations that have been linked to familial forms of PD, DNA polymorphisms at several genes could contribute to the risk of developing sporadic PD. The leucine-rich repeat kinase 2 gene (*LRRK2*, *PARK8*) has been linked to both sporadic and familial PD.<sup>2–5</sup> The *LRRK2* p.G2019S mutation accounts for 30–40% of PD cases among North African Berbers patients and Ashkenazi Jews and has also been found in 5% of Spanish patients.<sup>6</sup> The p.R1441G mutation is also common in Spain, with a frequency in PD cases close to 8% in some regions.<sup>7</sup> The *LRRK2* gene encodes a protein with multiple functional domains, including leucine-rich repeats, a GTPase domain and a kinase domain, which, in addition to the brain, is expressed in multiple tissues.<sup>8</sup> The mutations found in PD patients disrupt the enzymatic activities of the protein by affecting the kinase (p.G2019S) or the GTPase (p.R1441G/C) activities.<sup>9,10</sup> Some studies also reported differences in *LRRK2* expression between brains from PD cases compared with those from controls.<sup>11–13</sup>

Most of the mutational screenings were focused on the *LRRK2* coding exons, whereas little is known about the contribution of 3'UTR variants to the risk for PD. However, several studies have

provided strong evidence of the role of this gene region on the regulation of *LRRK2* expression through microRNA binding, and the control of cellular pathways in which this gene is implicated. Among others, pathogenic *LRRK2* antagonizes let-7 and miR-184-3p in *Drosophila*, leading to the overproduction of proteins implicated in cell cycle regulation and survival.<sup>14</sup> It was also reported that miR-205 is able to suppress the expression of *LRRK2* by targeting a conserved binding site in the 3'UTR region.<sup>15</sup> Our first aim was to characterize the contribution of the *LRRK2* 3'UTR variation in the development of PD.

The study involved a total of 743 PD patients and 523 healthy controls, all Iberians from the region of Asturias (Northern Spain). The main characteristics of these cohorts are summarized in Table 1 and were previously reported.<sup>16</sup> The *LRRK2* 3'UTR was amplified in six overlapping fragments (Supplementary Table 1), which were subjected to single strand conformation analysis and direct sequencing to identify the nucleotide changes. We found a total of 12 variants (Supplementary Table 2), and only the SNP rs66737902 was linked to the risk of developing PD: allele C-carriers were frequent among the patients ( $P=0.005$ ; odds ratio = 1.47, 95% confidence

<sup>1</sup>Genética Molecular-Laboratorio de Medicina, Hospital Universitario Central de Asturias, Oviedo, Spain; <sup>2</sup>Department of Medicine, University of Oviedo, Oviedo, Spain; <sup>3</sup>Neurología, Hospitales Universitario Central Asturias and Álvarez Buylla-Mieres, Asturias, Spain; <sup>4</sup>Geriatric Research, Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, WA, USA and <sup>5</sup>Department of Neurology, University of Washington School of Medicine, Seattle, WA, USA  
Correspondence: Dr V Alvarez, Genética Molecular-Laboratorio de Medicina, Hospital Universitario Central de Asturias, 33006 Oviedo, Spain.  
E-mail: victoria.alvarez@sespa.prncast.es

**Table 1** Main characteristics of patients and controls enrolled in the study

	Patients (N = 743)	Controls (N = 523)
Mean age, years	67 ± 10	67 ± 12
Mean onset age, years	60 ± 12	—
Male	389 (52%)	261 (50%)
Early onset PD (≤50 years)	129 (17%)	—
Family history of PD	208 (28%)	—
<i>LRRK2</i>		
p.G2019S	16 (2%)	—
p.R1441G	15 (2%)	—

Abbreviations: LRRK2, leucine-rich repeat kinase 2 gene; PD, Parkinson's disease.

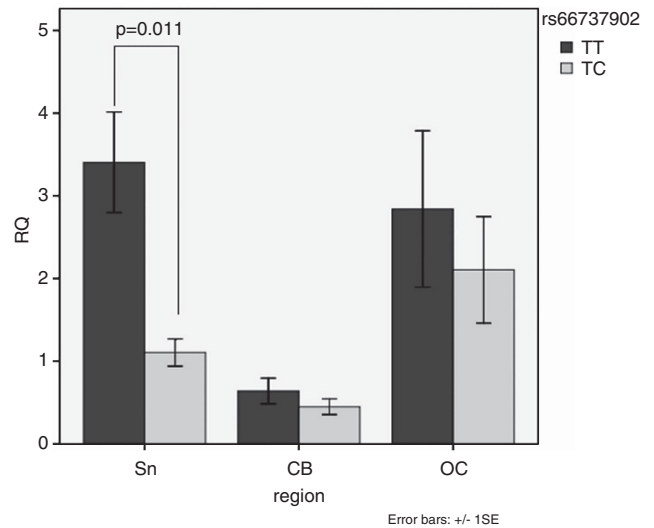
**Table 2** Genotype and allele frequencies of the four 3'UTR SNPs in PD patients and controls, and frequencies in the 1000 Caucasian genomes (www.ensembl.org)

	Patients (N = 743)	Controls (N = 523)	1000 genomes Europeans
<i>rs66737902</i> <sup>a</sup>			
TT	543 (73%)	418 (80%)	77%
TC	183 (25%)	94 (18%)	20%
CC	17 (2%)	11 (2%)	3%
T	1269 (0.85)	930 (0.89)	0.87
C	217 (0.15)	116 (0.11)	0.13
<i>rs10878441</i>			
AA	89 (12%)	63 (12%)	11%
AC	347 (47%)	260 (50%)	41%
CC	307 (41%)	200 (38%)	48%
A	525 (0.35)	282 (0.38)	0.32
C	961 (0.65)	452 (0.62)	0.68
<i>rs3886747</i>			
CC	89 (12%)	63 (12%)	11%
CT	347 (47%)	260 (50%)	41%
TT	307 (41%)	200 (38%)	48%
C	525 (0.35)	282 (0.38)	0.32
T	961 (0.65)	452 (0.62)	0.68
<i>rs1365770</i>			
GG	77 (10%)	42 (8%)	11%
GC	345 (47%)	251 (48%)	41%
CC	321 (43%)	230 (44%)	48%
G	499 (0.34)	335 (0.32)	0.32
C	987 (0.66)	711 (0.68)	0.68

Genotype frequencies did not deviate from the expected according to the Hardy-Weinberg equilibrium in both patients and controls.

<sup>a</sup>Patients vs controls, TC + CC vs TT:  $P=0.005$ , odds ratio = 1.47, 95% CI = 1.12–1.92 ( $P=0.02$ , odds ratio = 1.37, CI = 1.04–1.80 after excluding the 31 LRRK2 mutation carriers).

interval (CI) = 1.12–1.92) (Table 2). The sample size was enough for a statistical power >80%. The difference remained significant after correcting for age and gender ( $P=0.01$ ; odds ratio = 1.47, 95% CI = 1.09–1.97). To our knowledge, the association between PD and rs66737902 has not been reported by others who also examined the LRRK2 3'UTR.<sup>4,17</sup> This SNP showed a heterogeneous worldwide frequency distribution, but was absent among the Chinese and Japanese (www.ensembl.org). Differences in ethnic background could thus explain these discrepancies.



**Figure 1** Mean LRRK2 expression (cDNA) in the SN, cerebellum (CB) and occipital cortex (OC) of the nine PD donors according to the rs66737902 genotype (4 TC, 5 TT). The level of LRRK2 mRNA was determined through real-time PCR using ABI7500 equipment (Life Technologies, Carlsbad, CA, USA) and the GAPDH gene was used as the endogenous control for quantification. Each cDNA was amplified in triplicate using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) under the following PCR conditions: 95°C for 30s followed by 40 cycles at 95°C for 15s and at 58°C for 34s, and a final melting curve stage. The amount of LRRK2 was calculated as the fold difference  $2^{-\Delta\Delta Ct}$ , in which  $\Delta Ct = Ct\ LRRK2 - Ct\ GAPDH$ ;  $\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ controls\ mean$ . LRRK2 primers: ATA TAA AGG CTC GCG CTT CT and TGG CAT TCA CAA AGT GGT AAT (amplified fragment = 162 bp). GAPDH primers: GGA AGG ACT CAT GAC CAC AG and TTG GCA GGT TTT TCT AGA CGT (amplified fragment = 249 bp).

We found a significant linkage disequilibrium between rs66737902 and p.R1441G (rs33939927): a total of 15 patients were heterozygous for this mutation, and 14 and 1 were rs66737902 TC and TT, respectively. This mutation was not found among the 105 controls who were rs66737902 C carriers. After excluding the 15 p.R1441G mutation carriers, carriers of rs66737902 C remained significantly increased among the patients. This suggested that the risk effect of this 3'UTR allele could not be fully explained by its linkage disequilibrium with the LRRK2 mutation. In contrast, most of the p.G2019S mutation carriers (13/16) were homozygous for the rs66737902 TT protective genotype.

We measured the LRRK2 cDNA levels in three brain regions (SN, cerebellum, and occipital cortex) of 9 PD donors kindly provided by the London Neurodegenerative Diseases Brain Bank (Supplementary Table 3). Tissue processing and expression assays were performed as reported by Cardo et al.<sup>16</sup> Briefly, the level of LRRK2 and GAPDH (constitutively expressed normalization control) cDNA was determined using SYBR Green. The amount of LRRK2 was calculated as the fold difference,  $2^{-\Delta\Delta Ct}$ . We found a significant lower mean level of LRRK2 cDNA ( $P=0.01$ ) in the SN of rs66737902 TC ( $n=4$ ) vs TT ( $n=5$ ) donors (Figure 1). This effect was not seen in the cerebellum and cortex samples. Some studies have found significant reductions of LRRK2 mRNA in the brains from PD cases compared with controls.<sup>11</sup> In the brains of patients with PD, LRRK2 immunoreactivity was reduced in SN neurons, a fact that reflects the disease-associated loss of dopaminergic neurons in this brain region.<sup>12</sup> Another study did not find significant alteration in LRRK2 expression between brain tissue from p.G2019S mutation carriers and those from controls.<sup>13</sup> None of the nine brain donors analyzed was a p.R1441G

carrier, and thus we could not examine whether this mutation was also linked to differences in *LRRK2* expression. The differences in mRNA expression between the genotypes could be attributed to its linkage disequilibrium with p.R1441G, but whether they also exist at the protein level can be confirmed only through additional studies.

Finally, an online analysis with two miRNA-site prediction software programs (<http://www.microrna.org>; <http://diana.cslab.ece.ntua.gr/>) identified miR-138.2 as a candidate that could bind to the rs66737902 T sequence (Supplementary Figure 1). We found that this miRNA was expressed in all the nine SN samples (Supplementary Figure 2). Thus, the PD-protective T allele could result in enhanced miRNA binding and downregulation of the *LRRK2* mRNA. However, this would be in opposition to the higher transcript levels among the TT SN samples. Moreover, this binding site was recognized by only one of the programs and with a low score (Supplementary Figure 1). Although we considered unlikely a direct effect of the rs66737902 SNP through different binding of miR-138.2 to the two alleles, *in vitro* studies with neural cells transfected with reporter vectors coupled to 3'UTRs with the two alleles are required in case other studies confirm the genetic association.

In conclusion, we report the association between the *LRRK2* rs66737902 SNP and PD. This effect could be mediated by a significant reduction of gene expression in the SN of individuals with the risk allele, and require validation in other populations with larger samples of brain tissues.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### ACKNOWLEDGEMENTS

We thank Dr Claire Troakes and the London Neurodegenerative Diseases Brain Bank for supplying all post mortem brain samples. We also thank the 'Fundacion Parkinson Asturias' and 'Obra Social Cajastur' for their support. This work was supported by grants from the Spanish 'Fondo de Investigaciones Sanitarias-Fondos FEDER' European Union (FIS 11/0093). LFC is a predoctoral fellowship from FICYT-Principado de Asturias.

**Author contributions:** All the authors contributed to this work by recruiting the patients and obtaining the clinical and anthropometric data or by performing the laboratory work. All authors have read and approved the submission of this manuscript.

- de Lau, L. M. & Breteler, M. M. Epidemiology of Parkinson's disease. *Lancet Neurol.* **5**, 525–535 (2006).
- Paisan-Ruiz, C., Jain, S., Evans, E. W., Gilks, W. P., Simon, J., van der Brug, M. *et al.* Cloning of the gene containing mutations that cause *PARK8*-linked Parkinson's disease. *Neuron* **44**, 595–600 (2004).
- Healy, D. G., Falchi, M., O'Sullivan, S. S., Bonifati, V., Durr, A., Bressman, S. *et al.* Phenotype, genotype, and worldwide genetic penetrance of *LRRK2*-associated Parkinson's disease: a case-control study. *Lancet Neurol.* **7**, 583–590 (2008).
- Rubio, J. P., Topp, S., Warren, L., St Jean, P. L., Wegmann, D., Kessner, D. *et al.* Deep sequencing of the *LRRK2* gene in 14,002 individuals reveals evidence of purifying selection and independent origin of the p.Arg1628Pro mutation in Europe. *Hum. Mutat.* **33**, 1087–1098 (2012).
- Seki, N., Takahashi, Y., Tomiyama, H., Rogaeva, E., Murayama, S., Mizuno, Y. *et al.* Comprehensive mutational analysis of *LRRK2* reveals variants supporting association with autosomal dominant Parkinson's disease. *J. Hum. Genet.* **56**, 671–675 (2011).
- Correia Guedes, L., Ferreira, J. J., Rosa, M. M., Coelho, M., Bonifati, V. & Sampaio, C. Worldwide frequency of G2019S *LRRK2* mutation in Parkinson's disease: a systematic review. *Parkinsonism Relat. Disord.* **16**, 237–242 (2010).
- Gonzalez-Fernandez, M. C., Lezcano, E., Ross, O. A., Gomez-Esteban, J. C., Gomez-Busto, F., Velasco, F. *et al.* *Lrrk2*-associated parkinsonism is a major cause of disease in Northern Spain. *Parkinsonism Relat. Disord.* **13**, 509–515 (2007).
- Cookson, M. R. The role of leucine-rich repeat kinase 2 (*LRRK2*) in Parkinson's disease. *Nat. Rev. Neurosci.* **11**, 791–797 (2010).
- West, A. B., Moore, D. J., Biskup, S., Bugayenko, A., Smith, W. W., Ross, C. A. *et al.* Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc. Natl Acad. Sci. USA* **102**, 16842–16847 (2005).
- Lewis, P. A., Greggio, E., Beilina, A., Jain, S., Baker, A. & Cookson, M. R. The R1441C mutation of *LRRK2* disrupts GTP hydrolysis. *Biochem. Biophys. Res. Commun.* **357**, 668–671 (2007).
- Sharma, S., Bandopadhyay, R., Lashley, T., Renton, A. E., Kingsbury, A. E., Kumaran, R. *et al.* *LRRK2* expression in idiopathic and G2019S positive Parkinson's disease subjects: a morphological and quantitative study. *Neuropathol. Appl. Neurobiol.* **37**, 777–790 (2011).
- Higashi, S., Biskup, S., West, A. B., Trinkaus, D., Dawson, V. L., Faull, R. L. *et al.* Localization of Parkinson's disease-associated *LRRK2* in normal and pathological human brain. *Brain Res.* **1155**, 208–219 (2007).
- Devine, M. J., Kaganovich, A., Rytten, M., Mamais, A., Trabzuni, D., Manzoni, C. *et al.* Pathogenic *LRRK2* mutations do not alter gene expression in cell model systems or human brain tissue. *PLoS One* **6**, e22489 (2011).
- Gehrke, S., Imai, Y., Sokol, N. & Lu, B. Pathogenic *LRRK2* negatively regulates microRNA-mediated translational repression. *Nature* **466**, 637–641 (2010).
- Cho, H. J., Liu, G., Jin, S. M., Parisiadou, L., Xie, C., Yu, J. *et al.* MicroRNA-205 regulates the expression of Parkinson's disease-related leucine-rich repeat kinase 2 protein. *Hum. Mol. Genet.* **22**, 608–620 (2012).
- Cardo, L. F., Coto, E., de Mena, L., Ribacoba, R., Mata, I. F., Menendez, M. *et al.* Alpha-synuclein transcript isoforms in three different brain regions from Parkinson's disease and healthy subjects in relation to the SNCA rs356165/rs11931074 polymorphisms. *Neurosci. Lett.* **562**, 45–49 (2014).
- Wu-Chou, Y. H., Chen, Y. T., Yeh, T. H., Chang, H. C., Weng, Y. H., Lai, S. C. *et al.* Genetic variants of SNCA and *LRRK2* genes are associated with sporadic PD susceptibility: a replication study in a Taiwanese cohort. *Parkinsonism Relat. Disord.* **19**, 251–255 (2013).

Supplementary Information accompanies the paper on Journal of Human Genetics website (<http://www.nature.com/jhg>)