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# Mutational Screening of PARKIN Identified a 3′ UTR Variant (rs62637702) Associated with Parkinson's Disease

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**Abstract** *PRKN* mutations have been linked to Parkinson's disease (PD). Most of the mutational screenings have focused on the coding exons. The 3′ untranslated region (UTR) could also harbor functionally relevant nucleotide changes. We performed a mutational screening of *PRKN* in a cohort of early-onset PD patients ($n$=235) from Spain. We found 16 mutations (five new): 16 patients (7 %) carried two mutations and only one mutation was found in 28 (12 %). Patients with two mutations had significantly lower mean age (30±9 years) compared to patients with one (40±7) or no mutation (42±7). We found a total of 15 nucleotide variants (three new) in the 3′ UTR region. The frequency of carriers of the rare *rs62637702* G allele (*94A/G) was significantly lower among the patients compared to healthy controls ($n$=418) (0.03 vs. 0.004; $p$<0.001), suggesting a protective role for this allele. In order to investigate the basal effect of this variant, we performed luciferase assays. No different basal activity was observed between the two

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alleles. In conclusion, the rs62637702 polymorphism was associated with PD. This could be a surrogate marker for disease risk, in linkage disequilibrium with other non-identified functional variant.

**Keywords** Parkinson's disease · Parkin · Mutation · DNA polymorphisms · Genetic risk · Genetics

## Introduction

Several genes have been linked to autosomal dominant and recessive forms of Parkinson's disease (PD) (Vila and Przedborski 2004). *PRKN* (*PARK2* locus, OMIM*602544) maps to chromosome region 6q35.3-q27.1 and encodes an E3 ubiquitin protein ligase. *PRKN* mutations have been found in 10–20 % of the patients with early-onset PD (EOPD), mainly homozygous or compound heterozygous for *PRKN* mutations (Alvarez et al. 2001; Hedrich et al. 2002). However, most of the mutational screenings also reported a large amount of patients with only one identified mutated allele. Based on these findings, a dominant effect with variable penetrance has been proposed for some *PRKN* mutations (Sun et al. 2006; Lesage et al. 2008). These mutational studies analyzed the coding exons and a few intron flanking nucleotides, and the presence of a second non-identified *PRKN* mutation outside this regions could not be excluded. Nucleotide changes in the 3′untranslated region (3′ UTR) of several genes have been linked to the risk of developing several diseases, including PD (Wang et al. 2008; Li et al. 2012; Papagregoriou et al. 2012). The pathogenic mechanism for these 3′ UTR variants would implicate the differential binding of micro RNAs (miRNAs) (Hebert and De Strooper 2007; Wang et al. 2008).

Our aim was to contribute in defining the mutational spectrum of *PRKN*. For this purpose, we analyzed the coding exons and the 3′ UTR in a cohort of Spanish PD patients.

## Materials and Methods

### Patients and Controls

We searched for *PRKN* mutations in a total of 235 EOPD patients (mean age at diagnosis 41±8 years; 65 % male) (Supplementary Table 1). PD was diagnosed according to the UK Parkinson's Disease Society Brain Bank Clinical Criteria (Hughes et al. 1992). EOPD was considered as an onset of symptoms at age ≤50 years (see www.ninds.nih.gov/disorders/parkinsons_disease). Patients who had at least one first-degree PD affected relative were classified as familial cases. The controls were 418 healthy individuals (140≤50 years; 278>50 years) from the same regions (Asturias and Navarre, Northern Spain). The rs62637702 single nucleotide polymorphism (SNP) was also genotyped in a total of 306 late-onset (LOPD) patients (mean onset age 66±9 years; 51 % male).

All the patients/controls gave their informed consent to participate in the study, approved by the Ethical Committees of Hospital Central Asturias and Clínica Universidad de Navarra.

### *PRKN* Sequencing

Genomic DNA was obtained from blood leukocytes. The 12 *PRKN* exons were polymerase chain reaction (PCR)-amplified in the 235 EOPD patients with primers designated from the intronic sequences (Supplementary Table 2). PCR fragments were sequenced with BigDye chemistry in an ABI3130xl equipment (Applied Biosystems, Foster City, CA, USA). All the new (non-reported) putative mutations were screened in the 418 healthy controls through single strand conformation analysis (SSCA) of the corresponding PCR fragments (Supplementary Fig. 1). The putative functional effect of intronic nucleotide changes on pre-mRNA splicing was determined online (http://www.fruitfly.org/seq_tools/splice.html).

### *PRKN* Exon Rearrangements

Patients with only one sequencing mutation ($n$=28) or no mutations ($n$=191) were screened for *PRKN* exon rearrangements through multiple ligation-dependent probe amplification (MLPA) assay (P052 and P052B SALSA MLPA Parkinson 2 probemix kit, MRC-Holland, Amsterdam, The Netherlands).

### *PRKN* 3′ UTR Analysis

The 3′ UTR of *PRKN* was amplified (ten overlapping PCR fragments) in all the patients and controls and the presence of DNA variants determined through SSCA (Supplementary Table 2 and Supplementary Fig. 1). Individuals with different SSCA patterns were sequenced to characterize the nucleotide variants. Two online tools (targetscan.org and microRNA.org) were used to determine whether SNP rs62637702 could affect a miRNA binding site.

### Luciferase Reporter Assay

Two luciferase reporter plasmids containing the PRKN 3′ UTR sequence and differing only by the rs62637702 SNP were constructed. The two reporter plasmids were constructed by inserting the PCR fragments from two patients with primers GGAGC*ACTAGT*ATACCCAGTGTC TACCTTCATTTT (forward) and CATAAGCTTGT GAGACTCATGCCCTCAGTT (reverse) into the SpeI/HindIII sites in the 3′ of the luciferase gene of the pmiR-

**Table 1** *PRKN* mutations found in the coding exons and intronic flanking nucleotides

| Mutation | Exon/intron | Variant ID | Type |
|---|---|---|---|
| p.Q34fs | Exon 2 | new | Frameshift |
| c.225delA | Exon 2 | known | Frameshift |
| Exon rearrangement | Exon 2 | known | Deletion |
| Exon rearrangement | Exon 3 | known | Deletion |
| Exon rearrangement | Exon4 | known | Deletion |
| Exon rearrangement | Exon2-3 | known | Deletion |
| Exon rearrangement | Exon 3-5 | known | Duplication |
| c.461delG | Exon 4 | New | Frameshift |
| M192L | Exon 5 | known | Missense |
| P202L | Exon 5 | New | Missense |
| C212Y | Exon 6 | Rs137853058 | Missense |
| T240M | Exon 6 | Rs137853054 | Missense |
| R275W | Exon 7 | Rs34424986 | Missense |
| c.1175-1176 delGA | Exon 11 | known | Frameshift |
| c.1203-1204 delTC | Exon 11 | New | Frameshift |
| R402C | Exon 11 | Rs55830907 | Missense |
| Ivs11 -3bpC/G | Intron 11 | known | Splicing[a] |
| G430S | Exon 12 | Rs191486604 | Missense |
| P437L | Exon 12 | Rs149953814 | Missense |
| W445.Stop | Exon 12 | known | Frameshift |
| V456I | Exon12 | New | Missense |

Report vector (Ambion, Austin, TX). Twenty-four hours before transfection $6\times10^4$ HEK 293 cells per well were plated in a 24-well plate. Each well was transfected with 150 ng of *PRKN*-3′ UTR-luciferase construct and 50 ng of Renilla control vector. After 48 h, the assays were performed using the Dual-Luciferase® reporter assay system (Promega Corp., Madison, WI). Basal luciferase activities were normalized to Renilla activities for each well transfected. The experiment was performed in triplicate, and each well was assayed in triplicate.

### Statistical Analysis

Differences between 3′ UTR allele and genotype frequencies were compared through Chi-squared or Fisher's exact tests. These were also used to determine whether the observed genotype frequencies differed from those expected under the Hardy–Weinberg equilibrium. The Student's *t* and ANOVA were used to compare the mean age between the groups. Basal luciferase activities were expressed as means ± SD and differences, compared through ANOVA. All the statistical analysis were performed with the *R-project* free software (http://www.R-project.org), and a $p$ value <0.05 was considered as statistically significant.

The linkage disequilibrium for 3′ UTR SNPs and difference between haplotype frequencies were determined as reported (Barrett et al. 2005; Gabriel et al. 2002).

## Results

### *PRKN* Mutations

The sequencing of the *PRKN* coding exons + intronic flanking nucleotides identified a total of 16 mutations, five of them new (Table 1). All the new mutations gave typical SSCA patterns that were absent in the controls (Supplementary Fig. 1). Allele and genotype frequencies did not differ between patients and controls for the two common missense polymorphisms S167N and D394N.

We found two mutations in 15 patients (6.4 %) and only one in 12 (5.5 %) patients (Supplementary Table 3). PD subjects with only one mutation or without an identified *PRKN* mutation were screened for large gene rearrangements. Duplications of exons 2, 3, 2-3, or 2-4, and deletions of exons 4 or 3-4 were found in 17 patients (Supplementary Fig. 2). Only one was compound heterozygous for an exon 4 deletion and a single nucleotide mutation (T240>M). Thus, we identified two *PRKN* mutations in 16 (7 %) and only one in 28 (12 %) patients. Patients with two *PRKN* mutations had a significantly lower mean onset age (30.25±8.8 years) compared to patients with only one (39.75±7.2 years; $p<0.001$) and without mutations (42.02±6.9 years; $p<0.001$). Two mutations were also significantly more frequent among familial vs. sporadic patients ($p=0.045$) (Table 2).

### *PRKN* 3′ UTR Analysis

We identified 15 nucleotide variants in the *PRKN* 3′ UTR, 12 of them reported SNPs (Supplementary Table 4). All

**Table 2** Mean age at diagnosis and known family history in patients according to the PRKN mutation status

| | Sex (%male) | Family history *N* (%) | *p* value[a] | Mean age | *p* value[b] |
|---|---|---|---|---|---|
| Non-mutated (*N*=191) | 122 (63.9) | 59 (30.9) | | 42.02±6.9 | |
| Homozygous (*N*=16) | 13 (81.3) | 9 (56.3) | 0.045 | 30.25±8.8 | <0.001 |
| Heterozygous (*N*=28) | 17 (60.7) | 12 (42.9) | 0.210 | 39.75±7.7 | 0.36 |

[a] Non-*PRKN* mutation carriers vs. mutation carriers

[b] Mean age non-mutation carriers vs. mutation carriers

**Table 3** Rs62637702 genotype and allele frequencies in patients and healthy controls

| | EOPD ($n$=235) | Controls ≤50 years $N$=140 | LOPD ($n$=306) | Controls >50 years $N$=278 | Total patients* $N$=541 | Total controls* $N$=418 |
|---|---|---|---|---|---|---|
| AA | 233 (99 %) | 135 (96 %) | 303 (99 %) | 261 (94 %) | 536 (99 %) | 396 (95 %) |
| AG | 2 (1 %) | 5 (4 %) | 3 (1 %) | 17 (6 %) | 5 (1 %) | 22 (5 %) |
| GG | 0 | 0 | 0 | 0 | 0 | 0 |
| A | 468 (99 %) | 275 (98 %) | 609 (99 %) | 539 (97 %) | 1,072 (99 %) | 814 (97 %) |
| G | 2 (<1 %) | 5 (2 %) | 3 (<1 %) | 17 (3 %) | 5 (<1 %) | 22 (3 %) |

*$p$<0.001 (G carriers, total PD vs. total controls)

these variants gave a characteristic SSCA pattern and were thus SSCA-genotyped in the healthy controls. Two of the 3′ UTR variants (*93-94 Ins AC and *180-238 duplication) were found in two different patients (negative for *PRKN* mutations) and none of the controls. Only the rs62637702 (*94A/G) allele and genotype frequencies were significantly different between the two groups. Compared to EOPD patients, carriers of the rare G allele were more frequent in age-matched controls (≤50 years) and total controls, suggesting a protective effect against EOPD in our population (Table 3). To determine whether this SNP was also associated with the risk of developing LOPD, we genotyped 306 patients >50 years (onset age) and found that G-carriers were also more frequent in healthy controls than in LOPD (22/418, 5.3 % vs. 3/306, 1 %; $p$=0.001). No significant difference was found between the younger (≤50 years) and older (>50 years) controls. None of the G-carrier patients had *PRKN* mutations.

We determined the linkage disequilibrium between rs62637702 and the other 3′ UTR SNPs in our population (Supplementary Fig. 3). No significant difference was found for the haplotype frequencies, patients vs. controls (data not shown).

We used two public online tools (targetscan.org and microRNA.org) to determine whether this SNP could affect a miRNA binding site, and none of them predicted that any putative miRNA site could be affected by this SNP. To determine whether rs62637702 could affect basal gene expression, we cloned *PRKN* 3′ UTR fragments containing either A or G in luciferase reporter plasmids and transfected and measured the luciferase activity in HEK-293 cells. No significant differences were found between the two constructs (Fig. 1).

## Discussion

We reported the mutational spectrum of *PRKN* in a cohort of Spanish EOPD patients. In addition to sequencing the whole coding exons and immediately flanking intronic nucleotides,

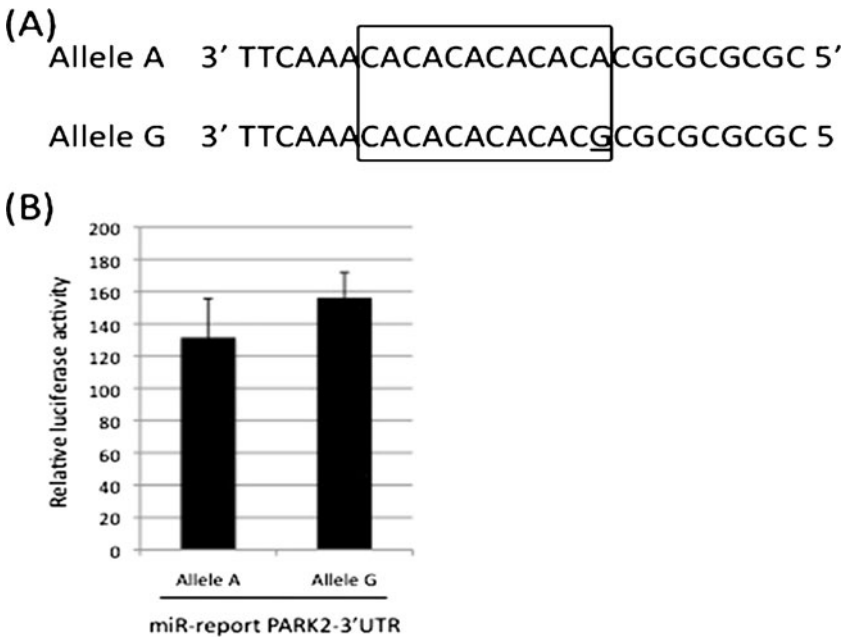


**Fig. 1** Relative luciferase expression of the two 3′ UTR constructs for rs62637702 alleles. **a** Scheme of the PARK2 3′ UTR-luciferase constructs containing the two rs62637702 alleles. **b** HEK 293 cells where transfected with the two constructs and harvested 48 h after transfection. Luciferase activity was normalized with Renilla activity. $p$=0.4

we also performed large gene duplication/deletion scanning. Our study was, thus, more complete than others that did not determined the presence of gene rearrangements. The frequency of patients with two (7 %) and one (12 %) mutation was similar to that reported by others (Hedrich et al. 2002; Oliveira et al. 2003; Periquet et al. 2003; Sironi et al. 2008; Koziorowski et al. 2009). In agreement with other studies, we also found a significantly lower mean onset age among patients with two mutations (Sironi et al. 2008).

To our knowledge, this is the first report describing the *PRKN* 3′ UTR variation in a cohort of EOPD patients. Because they did not encode for amino acids or affect pre-mRNA splicing, the 3′ UTRs have been rarely included in mutational screenings. This scenario has changed after the discovery of its role in translational regulation by microRNA (miRNA) binding. Through this mechanism, miRNAs regulate many cellular processes, and the deregulation of miRNA pathways could result in neurodegeneration and PD (Kosik 2006; Hebert and De Strooper 2007; Wang et al. 2008; de Mena et al. 2010). Some miRNAs have been implicated in the post-transcriptional regulation of PD-associated genes such as *SNCA*, and common 3′ UTR variants in other genes could regulate the risk for PD (Junn et al. 2009; Doxakis 2010).

Only two rare 3′ UTR changes (*93-94 Ins AC and *180-238 duplication) were found in two patients and absent among the controls. The main novelty in our study was a significant association between a rare SNP (*94A/G; rs62637702) and PD. G-carriers were more frequent among healthy controls, suggesting that this allele could have a protective effect against PD in our population. The protective effect was also supported by the higher frequency of the G-allele carriers among elderly compared to the younger controls (6 vs. 4 %), although the difference was non-significant. Sironi et al. found the rare G variant in three out of 146 EOPD (2 %) and two out of 50 controls (4 %) (Sironi et al 2008). Patients and controls in this study had a younger mean age compared to ours, and only 50 controls were genotyped by these authors. This could explain the differences in *94 G frequencies between the two studies. Bardien and colleagues also found this variant in EOPD, but the frequency in controls was not determined (Bardien et al. 2009).

The *94 A/G SNP could be directly linked to PD risk through a functional effect on parkin expression mediated by changes in a miRNA binding site. However, none of the two alleles was predicted to affect the binding of miRNAs. In addition, we did not find significant differences in luciferase basal activities between plasmid constructs differing by rs62637702. The lack of a direct effect for this SNP suggested that other gene variants in linkage disequilibrium with rs62637702 could explain the observed association with PD risk. Because no missense polymorphism linked to EOPD was identified, we can conclude that the observed effect of this SNP was due to linkage disequilibrium with a functional variant in the coding region. However, we cannot exclude that rs62637702 was a surrogate marker for a functional variant in the promoter or internal intronic nucleotides.

In conclusion, we reported the mutational spectrum of *PRKN*/*PARK2* gene in a cohort of Spanish EOPD patients. In addition, our screening of the 3′ UTR identified a significant association of SNP rs62637702 with the risk of developing PD. Further studies should be necessary to confirm this association in other populations and to clarify the functional effect of this and other linked *PRKN* variants.

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**Conflict of Interests** All the authors declare no conflicts of interest related with this work.

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