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MOVEMENT DISORDERS - SHORT COMMUNICATION

# Mutational screening of the mortalin gene (HSPA9) in Parkinson's disease

**Lorena De Mena · Eliecer Coto · Elena Sánchez-Ferrero · René Ribacoba · Luis M. Guisasola · Carlos Salvador · Marta Blázquez · Victoria Alvarez**

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**Abstract** Mortalin is a mitochondrial chaperone of the heat shock protein 70 family. Mortalin plays a central role in mitochondrial biogenesis through its capacity to direct the import of nuclear-encoded proteins into the mitochondria. As mitochondrial dysfunction has been involved in Parkinson's disease (PD), changes in mortalin function and expression could manifest as a higher risk of developing PD. In agreement with this, mortalin expression was decreased in the mitochondrial fraction of neurons from the *substantia nigra* of PD patients. We hypothesised that DNA variants in the mortalin gene (HSPA9) could contribute to the risk of developing PD. We analysed the 17 HSPA9 coding exons in 330 PD patients and 250 controls. In addition to several polymorphisms, found in patients and controls, three variants were found in 3 patients but none of the controls: two missense (R126 > W and P509 > S) and a 17 bp insertion in intron 8 (predicted to affect RNA splicing). Our study suggests that putative mutations in the mortalin, although rare, could contribute to the risk of developing PD.

**Keywords** Parkinson's disease · Mortalin · Mutations · Genetic risk

L. De Mena · E. Coto · E. Sánchez-Ferrero · V. Alvarez (✉)
Genética Molecular, Hospital Central de Asturias-Maternidad, 33006 Oviedo, Spain
e-mail: victoria.alvarez@sespa.princast.es

R. Ribacoba · L. M. Guisasola · C. Salvador · M. Blázquez
Neurología, Hospitales Central Asturias and Alvarez Buylla-Mieres, Asturias, Spain

## Introduction

Mitochondria perform the metabolic reactions necessary to generate energy as adenosine triphosphate (ATP). Mitochondrial dysfunction could be involved in Parkinson disease (PD) (Autere et al. 2004; Dodson and Guo 2007; Greenamyre et al. 1999; Parker Jr et al. 1989). Inhibition of complex I by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to the selective degeneration of dopaminergic neurons (Ekstrand et al. 2004; Falkenberg et al. 2002). A reduced activity of mitochondrial complex I has been found in patients with PD (Gaspari et al. 2004; Shapira 1998; van der Walt et al. 2003). Mice with a conditional deletion of the mitochondrial transcription factor A gene (tfam) showed Parkinson-like motor disabilities, which were relieved by L-dopa (Ekstrand et al. 2007). Some of the nuclear genes that have been linked to familial forms of PD are implicated in mitochondrial function (Canet-Aviles et al. 2004; Dachsel et al. 2006; Mata et al. 2006).

Mitochondria contain their own DNA in a single circular chromosome (mtDNA). The variation in the mtDNA, either as rare mutations or as common DNA-polymorphisms could reduce the capacity to produce ATP, and this impairment in energy supply could affect the function of neurons and other cells, increasing the risk for developing PD (Gu et al. 1998; Huerta et al. 2005; Simon et al. 2000). Most of the mitochondrial proteins are encoded by genes in the nucleus and need to pass through the outer and inner membrane channels, a process that requires the unfold/refold of the native conformation and is directed by chaperones (Deocaris et al. 2006). Mortalin is a mitochondrial chaperone that plays a central role in mitochondrial biogenesis, importing and partitioning the nuclear-encoded proteins within the two mitochondrial membranes and the matrix.

Mortalin is a member of the heat shock protein 70 (Hsp70) family of proteins, and is the only ATPase component of the mitochondrial import complex (Brunner et al. 1995; Schneider et al. 1994). Mortalin has an important anti-apoptotic function, and the overexpression of mortalin suppresses the pro-apoptotic effect of various substances. A decreased expression of mortalin has been related with the toxic effect of rotenone on dopaminergic neurons. Moreover, mortalin expression was significantly decreased in pathologically verified PD brains, compared to samples from healthy controls (Jin et al. 2006). These and other evidences suggested that mortalin plays an important role in the pathogenesis of PD, and mortalin dysfunction could contribute to the development of this common neurodegenerative disease (Shi et al. 2008).

In this report, we analysed the 17 coding exons of the mortalin gene (also known as HSPA9) in a cohort of PD patients and healthy controls. Our objective was to determine whether HSPA9 variants contributed to the risk for PD.

## Methods

### Patients and controls

The study included 330 patients (mean age 53 ± 11 years; 47% females) recruited in the period 2002–2007 by Neurologists from the Movement Disorder Units of Hospital Central Asturias and Hospital A. Buylla-Mieres, Spain. A total of 221 patients (67%) had a late-onset PD (age at the onset of symptoms >50 years; see http://www.ninds.nih.gov/disorders/parkinsons_disease for the definition of early and late-onset PD). A family history of PD, defined as the existence of at least one-first degree affected relative, was found in 18% of the cases.

A total of 250 healthy individuals were recruited as population controls (mean age 58 ± 14 years; 45% females). These controls were from the general population or spouses of the patients, and did not have symptoms of PD or any other neurodegenerative disorder. All the patients and controls were Spanish Caucasians from the region of Asturias (Northern Spain), and signed an informed consent to participate in the study, approved by the Ethical Committee of Hospital Central Asturias.

### Genetic analysis of mortalin/HSPA9

The DNA of patients and controls was obtained from blood leucocytes, and fragments corresponding to the 17 coding exons of HSPA9 were polymerase chain reaction (PCR) amplified with primers designated from the intronic flanking regions (Table 1). The HSPA9 sequence was obtained form the ENSEMBL database (www.ensembl.org ; accession number ENSG00000113013). In order to define the common variation of HSPA9 in PD patients, we followed two steps: first, we sequenced the 17 exons from 50 randomly chosen patients; second, the 17 PCR-fragments from the 330 patients and the 250 controls were analysed through single strand conformation analysis (SSCA). Briefly, 2 µl of each PCR were mixed with 10 µl of formamide and denatured for 5 min at 95°C, and 8 µl were

**Table 1** Primers used to amplify the 17 Mortalin/HSPA9 exons, and annealing temperature and size of the PCR-fragments

| Exon | Forward (5′ > 3′) | Reverse (5′ > 3′) | Annealing (°C) | Size (bp) |
|---|---|---|---|---|
| 1 | TCGAGTATCCTCTGGTCAGG | CTGGAGAATTCAAACCCTAAA | 54 | 374 |
| 2 | ACTAAGTGAGCCCAAGTTTT | GGAAAATTAACAGTGGTATACTAC | 58 | 279 |
| 3 | CTTCCAGTGACTTTGGTCTATC | CAGGTTCTCAAATTCCTTAACTA | 58 | 237 |
| 4 | GCGACAGAGTGAGACTCCAT | CCTCATCAACATGCTGAGG | 58 | 341 |
| 5 | TGGATGTTCATGGTATGTGG | GAGCACAGGGAGCTAGTGAT | 58 | 281 |
| 6 + 7 | GGATGACACAGGTTGTTGG | GACACTTTAGTTTCACACATCCA | 58 | 395 |
| 8 | GGTTATTGAAATTTATATCCTGGT | TCGTAAACAAAGCAAAGAGC | 58 | 325 |
| 9 | ACCTCACTAATGCTGGTGTTT | AAACTCAACTGACATTAGGCC | 58 | 239 |
| 10 | AATAAATGTAACCGTCATTTGG | CACCTGTCCCAAGAATACACT | 58 | 337 |
| 11 | GTTCTGCTCAGGTAGTTCCC | AACCCATGTGACAAGGTAGG | 60 | 322 |
| 12 | TTAGGAGTCTTCCAGTCTTGG | TCTCACTTTACAGTGAAGGAGG | 58 | 263 |
| 13 | TGGAAATAATAGCACTGGACC | AGAGGACTCATCATTCTAAGAGG | 58 | 262 |
| 14 | GTACTCTTAATGCAATGGAGGA | CCAAACTCCCACTGTCAAG | 58 | 238 |
| 15 | GTCTGAGTATAGACATTCAGCAAA | ATTAAACCCCTATCAAAACCC | 58 | 258 |
| 16 | CTACTTGTAATGGTACAGGAGACC | CCACCAGTGACAGTCATCAA | 58 | 273 |
| 17 | CCTCATCTTGGAGTGCAAC | CATTTCTGCTCAGGAAGTCTC | 58 | 232 |

Primers were designated from the intronic flanking sequences

loaded on polyacrylamide gels. After electrophoresis, the gels were silver-stained to visualise the electrophoretic patterns. For those PCR-fragments showing different SSCA electrophoretic patterns, we characterised the nucleotide changes responsible for the variation through direct sequencing on an ABI3130 system, using BigDye chemistry (Applied Biosystems, Foster City, CA, USA).

### Genotype frequencies and statistical analysis

Since each allele gives a characteristic SSCA-pattern, we could determine the genotype for each variant in all the patients and controls. Allele and genotype frequencies were compared between patients and controls through a Chi-squared test. All statistical analyses were performed with the SPSS statistical package.

## Results

Direct sequencing of the 17 HSPA9 exons in 50 patients revealed a total of four variants (Table 2). All of them had been previously reported as polymorphisms. As they were also identified in the SSCA of the 330 patients and 250 controls, we could genotype each patient and control for the 4 variants. Allele and genotype frequencies did not differ between the two groups, suggesting that common HSPA9 polymorphisms did not contribute to the risk of developing PD in our population (Table 2).

The SSCA also identified individuals with electrophoretic patterns different from those of the four known polymorphisms (Figure 1). After direct sequencing, we identified 10 non-previously reported nucleotide variants (Table 2). Two were missense changes, in exons 4 (R126 W) and 13 (P509S), and were found in sporadic cases, with onset ages of 73 years (126 W) and 61 years (509S). None of the controls carried these two changes.

For the seven new intronic variants, we determined the putative effect on RNA splicing through the BDPG software (www.fruitfly.org/seqtools/splice.html). Only the 17 bp insertion in intron, eight was predicted to affect the RNA splicing. This insertion was found in a patient with an onset age of 51 years and without family history of PD.

## Discussion

In this study, we determined the variation at the Mortalin/HSPA9 gene in a cohort of PD patients. Mortalin expression was decreased in the mitochondrial fraction of neurons from the *substantia nigra* of PD patients, compared with controls (Jin et al. 2006; Shi et al. 2008). Since the precise mechanism by which mortalin produces its effects is unknown, we cannot foresee the mechanism by which HSPA9 mutations could cause PD. However, mortalin associated with DJ-1 and α-synuclein, mutations of which cause familial PD (Dodson and Guo 2007; Jin et al. 2007). HSPA9 mutations might accomplish their effects by affecting the interaction of mortalin with DJ-1 and α-synuclein. Amino acid 126 lies in the actin-like ATPase domain of the protein, while amino acid 509 is in the heat shock protein peptide-binding domain. Functional studies to demonstrate that R126 > W and P509 > S affect mortalin function are necessary to confirm them as mutations likely linked to PD. However, in favour of a functional role for these two variants is the fact that the two amino acids are conserved between HSP70

**Table 2** Mortalin/HSPA9 variants and allele frequencies in patients and controls

| Polymorphism ID | Nucleotide change | Frequencies | |
|---|---|---|---|
| | | Patients | Controls |
| New | 5′UTR −34 $A > T$ | $T < 0.02$ | $T < 0.03$ |
| New | Intron 1 ivs. −56 $G > A$ | $A < 0.02$ | $A < 0.02$ |
| New | Intron 1 ivs. −51 ins/del AA | Del AA < 0.05 | Del AA < 0.05 |
| New | Intron 1 ivs. −58 ins/del GA | Del GA < 0.01 | Ins GA < 0.02 |
| New | Intron 2 ivs. +75 ins/del GAA | Del GAA = 0.34 | Del GAA = 0.31 |
| New[a] | Exon 4 c. 376 $G > A$, R126 W | $A < 0.02$ | $A = 0$ |
| New | Intron 7 ivs. −17 ins/del $A$ | Del $A = 0.47$ | Del $A = 0.46$ |
| rs41295717 | Exon 8 c. 775 $C > T$, A225A | $T = 0.02$ | $T = 0.01$ |
| New[b] | Intron 8 ivs. +8 | Ins < 0.005 | Ins = 0 |
| | Ins/del **GTAACTCACCTCTCTCT** | | |
| rs1042665 | Exon 9 c. 949 $T > C$, K316K | $C = 0.28$ | $C = 0.24$ |
| New | Intron 11 ivs. −16 $C > T$ | $T < 0.02$ | $T < 0.03$ |
| New[c] | Exon 13 1526G > A, P509S | $A < 0.005$ | $A = 0$ |
| rs1042686 | Exon 16 1882G > A, S627S | $A = 0.42$ | $A = 0.44$ |
| rs10117 | Exon 16 1933G > A, L645L | $A < 0.01$ | $A < 0.01$ |

Nucleotides in exons were numbered considering +1 as the first base in codon 1 of the transcript (ENST 00000297185; www.ensembl.org). Nucleotides in introns were numbered according to the genomic sequence (ENSG 00000113013)

[a] 126 W was found in one patient and none of the controls

[b] The 17 bp insertion (nucleotide sequence in bold) was in one patient and none of the controls

[c] 509S was found in one patient and none of the controls

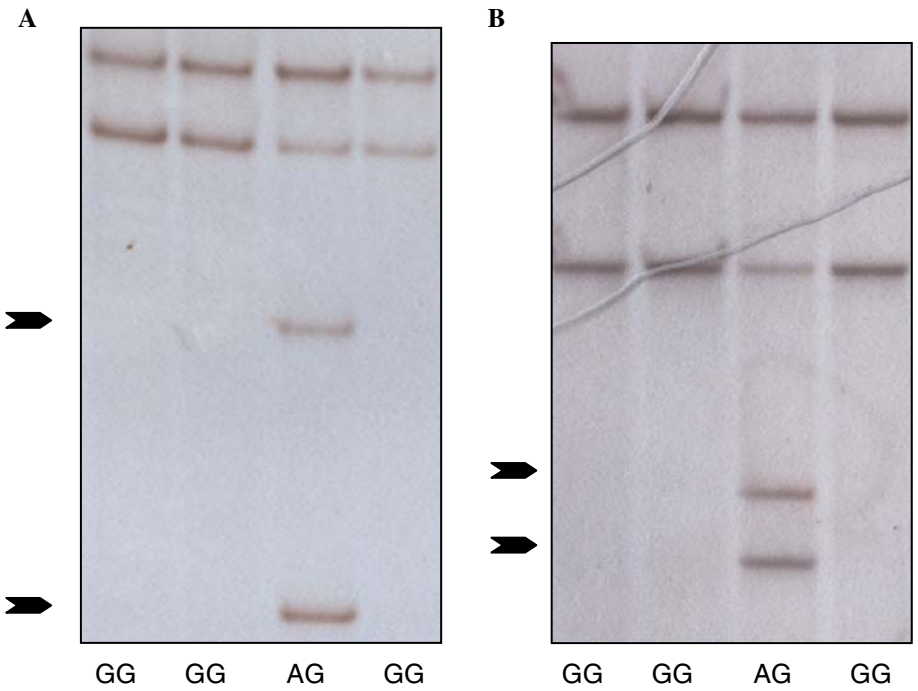


**Fig. 1** SSCA patterns corresponding to the normal and the two missense variants R126 W (**a**) and P508S (**b**). The *arrows* indicate the electrophoretic bands that characterise the two putative mutations

**Fig. 2** Partial sequence of mortalin, showing the conservation of human amino acid 126 and 508 (*arrows*) in mice, chicken and drosophila

| | | | |
|---|---|---|---|
| *H. sapiens* | 85 | RTTPSVVAFTADGERLVGMPAKRQAVTNPNNTFYATKRLIGRRY | 128 |
| *M. musculus* | 85 | RTTPSVVAFTSDGERLVGMPAKRQAVTNPNNTFYATKRLIGRRY | 128 |
| *G. gallus* | 87 | RTTPSVVAFTADGERLVGMPAKRQAVTNPHNTFYATKRLIGRRF | 130 |
| *D. melanogaster* | 84 | RTTPSHVAFTKDGERLVGMPAKRQAVTNSANTFYATKRLIGRRF | 127 |

| | | | |
|---|---|---|---|
| *H. sapiens* | 475 | AADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPPAPRGVPQI | 518 |
| *M. musculus* | 475 | AADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPPAPRGVPQI | 518 |
| *G. gallus* | 477 | AADGQTQVEIKVCQGEREMASDNKLLGQFTLVGIPPAPRGVPQI | 520 |
| *D. melanogaster* | 475 | ASDGQTQVEIKVHQGEREMANDNKLLGSFTLVGIPPAPRGVPQI | 518 |

members from different species (see the alignment containing sequences at http://www.bioinf.manchester.ac.uk/cgi-bin/dbbrowser/ALIGN; Fig. 2).

Our study has some limitations. First, the three putative mutations were found in patients with a late-onset PD and without affected relatives in their families. Although, they were absent among the 250 controls, we could not demonstrate a familial segregation of the disease with these HSPA9 putative mutations. Second, SSCA is an indirect technique for detecting nucleotide changes in PCR-amplified fragments, and the existence of nucleotide variants that are not detected (approximately 5% of false negatives) is the main limitation for this method. It is thus possible that some mutations/polymorphisms in the HSPA9 gene were not identified in our study.

In conclusion, we described the variation in the HSPA9/mortalin gene in a cohort of Parkinson's Disease patients. In addition to previously reported polymorphisms, we also found new variants. Two missense and one putative splicing variants were found in three patients and none of the healthy controls. Additional studies of the HSPA9 variation in large cohorts of patients/controls, and functional studies to define the effect of these putative mutations are necessary to confirm their role in PD.

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