

# Analysis of the *micro-RNA-133* and *PITX3* genes in Parkinson's Disease

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**Key words:** Parkinson's disease; micro RNAs; DNA polymorphisms; genetic risk.

**Running short title:** Micro RNA-133/PITX3 genes in Parkinson's disease.

**Disclosure:** The authors report no conflicts of interest.

**Authors contribution:** E.C. and V.A. designated the work, analyzed the results (including the statistics), and wrote the manuscript. L.M., L.F.C., M.D., and A.I.C., performed all the experiments. M.B., R.R., C.S., P.P., L.I.S., G.M., and M.M. recruited the patients and obtained all the clinical and anthropometric data.

**ABSTRACT (193 WORDS).**

MicroRNAs are small RNA sequences that negatively regulate gene expression by binding to the 3' untranslated regions of mRNAs. MiR-133b has been implicated in Parkinson's Disease (PD) by a mechanism that involves the regulation of the transcription factor PITX3. The variation in these genes could contribute to the risk of developing PD. We searched for DNA variants in miR-133 and *PITX3* genes in PD patients and healthy controls from Spain. We found common DNA variants in the three miR-133 genes. Genotyping of a first set of patients (n=777) and controls (n=650) showed a higher frequency of homozygous for a miR-133b variant (-90 del A) in PD-patients (6/575; 1%) than in healthy controls (0/650) (p=0.03). However, this association was not confirmed in a second set of patients (1/250; 0.4%) and controls (2/210; 1%). No common *PITX3* variants were associated with PD, although a rare missense change (G32S) was found in only one patient and none of the controls.

In conclusion, we report the variation in genes of a pathway that has been involved in dopaminergic neuron differentiation and survival. Our work suggests that miR-133 and *PITX3* gene variants did not contribute to the risk for PD.

**Main text: 2,640 words.**

## **INTRODUCTION.**

Micro RNAs (miRNAs) are 20-23 nucleotide RNAs that bind to the 3' untranslated regions (3'UTRs) of mRNAs, and regulate gene expression at the posttranscriptional level. By base pairing to mRNAs, miRNAs repress the expression of many genes and regulate cellular processes such as differentiation, growth, proliferation, and apoptosis [Valencia-Sánchez et al., 2006]. To date, more than 500 human miRNAs have been found (see [www. microrna.sanger.ac.uk](http://www.microrna.sanger.ac.uk)). The miRNA genes encode a large RNA transcript known as pri-miRNA [Lee et al., 2004]. Pri-miRNAs are processed to approximately 90 nucleotides pre-miRNAs, and these are in turn processed to the single-strand mature miRNA. Mature miRNAs are incorporated into the RNA induced silencing complex that regulates mRNA expression [Bartel 2004].

MiRNAs can play a role in dopaminergic neurons (DN) differentiation and survival [Hébert et al., 2007; Kosik 2006]. A quantitative analysis of 224 miRNAs identified a loss of miR-133b expression in PD subjects. This miRNA was specifically expressed in the midbrain of normal mice and markedly reduced in mice dopamine-deficiency models, and regulates the maturation and function of DNs within a negative feedback circuit that includes the transcription factor Pitx3 [Kim et al., 2007]. Recent reports have provided evidence for the association between *PITX3* polymorphisms and PD [Bergman et al., 2008; Fuch et al., 2009; Haubenberger et al., 2009; Le et al., 2009].

DNA variants at miRNA genes and at the 3' UTR regions where miRNAs bind have been associated with the risk for diseases [Duan et al., 2007; Li et al., 2009]. A DNA polymorphism in the 3'UTR of *FGF20* that disrupted a binding site for miR-433 and increased the translation of FGF20 was associated with PD [Wang et al., 2008]. However, this association was not detected by other authors [Clarimon et al., 2005; Wider et al. 2009].

We hypothesised that DNA variants in the miRNA-133 and *PITX3* genes could contribute to the risk for PD. We searched for DNA variants in these miRNA genes in PD-patients, and compared the genotype frequencies between patients and healthy controls.

## **METHODS.**

### **Patients and Controls.**

The study included a total of 1,027 Parkinson's patients (mean age at diagnosis  $58 \pm 13$  years; 52% male) recruited in the period 2002-2008 by Neurologists from the Movement Disorder Units of four reference Hospitals (Central de Asturias, Mieres, Avilés, and Clínica Universitaria Pamplona) from two regions in Spain: Asturias and Navarra. The control group consisted of 860 healthy individuals (mean age  $59 \pm 16$  years; 54% male). These controls were from the general population, Hospital staff, or spouses of the patients, and did not have symptoms of PD or any other neurodegenerative disorder. However, they were not subjected to a detailed examination by a specialist on movement disorders to exclude the existence of asymptomatic PD. The cohort from Asturias was composed by 777 patients (mean age at diagnosis  $58 \pm 13$  years) and 650 controls ( $58 \pm 18$  years), and the cohort from Navarra consisted on 250 patients (mean age at diagnosis  $57 \pm 12$ ) and 210 controls ( $71 \pm 9$  years) (**Table 1**). Patients and controls were Spanish Caucasians and signed an informed consent to participate in the study, approved by the Ethical Committee of Hospital Central Asturias.

### **Analysis of miRNA-133a-1, -133a-2, and -133b genes.**

Genomic DNA was obtained from 10 ml of blood, and 350 to 450 nucleotides of the miR-133a-1, 133a-2, and 133b genes were polymerase chain reaction (PCR)-amplified with primers derived from the corresponding sequences in the *Ensembl* database ([www.ensembl.org](http://www.ensembl.org)) (**Supplementary Table**). The genetic analysis was performed in two steps: search for DNA variants and genotyping of specific variants. To search for DNA variants the PCR fragments from 350 patients and 200 controls (randomly chosen from total patients and controls) were analysed through denaturing high performance liquid chromatography (DHPLC) in a Varian Helix system, and eluted with a linear binary gradient created with buffers Varian Helix A (triethylammonium amine – TEAA) and B (TEAA + 25% acetonitrile) ([www.varianinc.org](http://www.varianinc.org)). Primers and PCR and DHPLC conditions are available upon request to the corresponding author. To identify the nucleotide changes, fragments with multiple elution peaks were sequenced using BigDye chemistry in an automated ABI3130 capillary system (Applied Biosystems, Foster City, CA, USA).

### **Genotyping of miR-133 polymorphisms.**

To determine the association between three DNA variants in the miR-133a-1, -133a-2, and -133b, we genotyped 575 patients and 650 controls through PCR-RFLP: a PCR-fragment was digested with a restriction enzyme which target sequence was affected by the nucleotide change, followed by electrophoresis on 3% agarose gels to visualise the different alleles. Because we found an statistical trend toward the association of the miRNA-133b polymorphism and PD, we extended the genotyping of this variant to a total of 1,027 patients and 860 controls.

For the 133a1 +85 A/C variant the DNA was PCR amplified with primers CAGTGACTGAAGCATTGGTATGATAA (forward) and AAGTTATTTGATTATAATCACAGTCTGCTT (reverse). The reverse primer had a mismatched nucleotide in the penultimate position (T instead of A) to introduce an *AflIII* site (CTTAAG) on fragments with +85 C. After digestion with this enzyme and electrophoresis on agarose gels, alleles were visualised as fragments of 360 bp (A) or 330 + 30 bp (C). The 133a2 -191 G/A polymorphism was genotyped by digestion with *MspI* (CCGG) of a PCR fragment generated with primers 133a2 F and R (supplementary table). In addition to two constant fragments of 55 and 35 bp, alleles were visualised as bands of 354 bp (A) and 212 + 142 bp (G). The 133b -90 ins/del A was genotyped by *MboI* (GATC) digestion of PCR fragments generated with primers 133b F and R (supplementary table). Alleles were visualised as fragments of 306 bp (insA) and 231 + 75 bp (delA).

### ***PITX3* genotyping.**

We searched for DNA variants in the entire coding and 3'UTR sequence of *PITX3* (exons 2 to 4) in 350 patients and 250 controls through single strand conformation analysis (SSCA). Briefly, fragments 300 to 450 bp were amplified (see the primers in the supplementary **table**), and subjected to SSCA as reported [García-Castro et al., 2003; Gasser et al., 2007]. Fragments that showed different SSCA patterns were sequenced to determine the nucleotide variation. To determine the frequency of the G32S variant, SSCA of the exon 2 fragment was performed on 1,027 patients and 860 controls.

### ***LRRK2* R1441G and G2019S genotyping.**

All the patients were characterised for three *LRRK2* mutations: R1441G and R1441C in exon 31 and G2019S in exon 41 (Mata et al., 2006). Genomic DNA was PCR-amplified with primers that matched intronic sequences flanking exons 31 and 41 of *LRRK2*, followed by sequencing. Primers and PCR

and sequencing conditions for the analysis for these mutations are available upon request to the corresponding author.

### **Statistical Analysis.**

Allele and genotype frequencies in patients and controls were compared through a Chi-squared test. For those comparisons with any expected frequency below 1, we used the Yate's correction. The Chi-squared was also used to analyse the deviation from the Hardy–Weinberg equilibrium of the genotype frequencies. The ANOVA was used to compare the mean age between the groups. All statistical analyses were performed with the SPSS statistical package (v.11.0). A  $p < 0.01$  was considered as statistically significant.

## RESULTS

### MiRNA-133 variation.

DHPLC analysis of 350 PD-patients and 200 controls showed multiple elution peaks for the 133a-1, 133a-2, and 133b PCR-fragments. We found two new (non previously reported) polymorphisms: -90 ins/del A in 133b and +85 A/C in 133a-1. In the 133a-2 gene we found four previously reported single nucleotide polymorphisms (SNPs): -191 G/A (SNP rs45547937), -171G/A (rs13040566), -88 G/A (rs13040413), and +78 T/C (rs6062251). We sequenced 30 patients and 30 controls and found that they were homozygous for the -191/-171/-88 polymorphisms (-191AA/-171AA/-88AA, or -191GG/-171GG/-88GG), or heterozygous for these SNPs. This suggested that the three 133a-2 SNPs were in complete linkage disequilibrium (LD), defining two haplotypes: -191A/-171A/-88A and -191G/-171G/-88G. We found +78 T/C heterozygotes among individuals homozygotes for the -191G/-171G/-88G haplotype, but all the -191AA/-171AA/-88AA were also +78CC.

### MiRNA-133 genotype frequencies.

We genotyped three nucleotide variants in the miR-133a-1, 133a-2 (rs45547937), and 133b in 575 patients and 650 controls. Allele and genotype frequencies for the 133a-1 and 133a-2 SNPs did not differ between PD-patients and controls. Homozygotes for the miR-133b -90 delA were more frequent among the patients (6/575, 1% vs. 0/650;  $p=0.03$ ) (**Table 2**). This suggested that homozygosity for -90 delA could be a risk factor for PD. We extended the analysis of this variant to a total of 1,027 patients and 860 controls (including patients/controls from a second population) and we found two healthy controls (aged 66 and 78 years) who were delA-homozygotes. We evaluated these controls to exclude they had symptoms of PD. In addition, they did not have a family history of PD. Although the frequency of -90 delA/delA was higher among the patients, the difference did not reach statistical significance ( $p=0.03$ ) (**Table 3**).

To exclude that incomplete restriction enzyme digestion could result mistyping of some delA homozygotes (that should be genotyped as heterozygotes), we confirmed the genotype for all the delA/insA patients and controls by DHPLC analysis, obtaining a two elution peak that confirmed the heterozygosity for all these samples.

All the patients were genotyped for three *LRRK2* mutations associated with PD: R1441G, R1441C, and G2019S were found in 4% of the patients from Asturias and in 6% of the patients from Navarra. In Table 3 we summarised the 133b frequencies in patients with and without these *LRRK2* mutations.

### ***PITX3* variation.**

We searched for *PITX3* variants in a total of 350 patients and 250 controls. The full coding and 3'UTR sequence was amplified with primers that matched the intronic flanking regions, and subjected to SSCA. Fragments from exons 2 and 3 showed heterogeneous SSCA patterns, and after sequencing we characterised five nucleotide variants: c.94G>T (exon 2), G32>S), IVS.+6bp C>T (intron 2), IVS.+70bp G>T (intron 3), IVS.+71bp C>T (intron 3), and c.285 T/C (SNP rs2281983; exon 3, silent for I 95). The three intronic nucleotide changes were new and only found in one patient and one control. SNP rs2281983 had been previously described and allele and genotype frequencies did not differ between patients and controls, suggesting that this variant did not contribute to PD risk. In addition, rs2281983 allele and genotype frequencies did not differ between early-onset and late-onset patients, or between patients with and without a family history of PD (data not shown).

The exon 2 c.93G>T was a missense change (G32S) found in only one of the 1,027 patients (a 52 years old female, negative for the two *LRRK2* mutations, and without relatives affected by PD). Its absence among the 860 controls and the fact that 32G was conserved among species suggests a contribution of this *PITX3* change to PD in this patient, although functional studies should be necessary to confirm a functional effect.

### **DISCUSSION.**

Micro RNA 133b and *pitx3* have been involved in dopamine neurons survival in mice. MiRNA-133b was specifically expressed in midbrain DNs and was deficient in midbrain tissue from patients with PD [Kim et al., 2007]. MiRNA-133b binds to the 3' UTR of *pitx3*, a transcription factor that regulates the maturation and function of midbrain DNs. *Pitx3* in turn binds to the miRNA-133b promoter, enhancing its expression. This negative feedback circuit could be essential to maintain DNs survival, and its deregulation could result in behaviors related with dopaminergic loss, such as locomotion [Hébert et al., 2007; Kim et al., 2007; Kosik 2006]. The human genes that encode miRNA-133b and *PITX3* are thus candidates to modify the risk for PD. In this report we searched for DNA variants in the three miRNA-133 and the *PITX3* genes, and analysed the association between these gene variants and PD.

We first searched for DNA variants in PD patients and healthy controls, and found several nucleotide changes in *PITX3* and miRNA-133b, 133a-1, and 133a-2. We used DHPLC and SSCA to analyse the variation in PCR-fragments, two indirect techniques with a low rate for false negatives [Buyse et al., 2000; García-Castro et al., 2003; Gasser et al., 2007]. Thus, we cannot exclude that some DNA variants in the four genes were not identified in our study. We compared the allele and genotype frequencies for three miRNA-133 variants between PD patients and controls. Allele and genotype frequencies for two miRNA-113a-1 (+85 A/C) and 133a-2 (rs45547937) polymorphisms did not differ between the two groups. We cannot exclude an association between the three 133a-2 SNPs (rs13040566, rs13040413, and rs6062251) not genotyped in our patients and controls, although this is unlikely for the -171 G/A and -88 G/A that were in complete linkage disequilibrium with -191 G/A.

In a first comparison of 575 patients and 650 controls, homozygotes for the rare miRNA-133b -90 delA were significantly more frequent among the patients. However, this difference was based on the absence of -90 delA/delA among the controls. We extended the analysis to a second set of 452 patients and 210 controls, and we found also two -90 delA homozygotes among the controls. These homozygous controls were 66 and 78 years old, above the mean age of delA/delA patients (55 years; range 35-66) and they did not have symptoms of PD. Thus, although we found a higher frequency of the -90 delA/delA among the patients the difference with the controls was not significant, and this suggested that miRNA-133b variants did not contribute to the risk for PD.

Our search for *PITX3* variants identified five nucleotide variants. One was a reported SNP in exon 3 (rs2281983, silent for I 95I), and allele and genotype frequencies did not differ between patients and controls. Three intronic *PITX3* nucleotide changes were found in only one patient and one control, and a missense change in exon 2 (c.94 G>T, G32S) was found in only one patient and none of the controls. We determined the presence of this change in a total of 1,027 patients and 860 controls, and we did not find the rare c.94T in the controls. This, in addition to the conservation of 32G among species, suggested that 32S could be associated with PD in this patient.

Two recent reports described a positive associations between a SNP (rs3758549) in the *PITX3* promoter and PD [Fuchs et al., 2009; Haubenberger et al., 2009]. However, a third study failed to replicate this association [Bergman et al., 2008]. A study involving 265 patients and 210 controls reported an association between SNP rs2281983 and PD, and the association was more robust

among early onset patients [Le et al., 2009]. In our population, allele and genotype frequencies for this *PITX3* polymorphism did not differ between patients and controls, or between early-onset and late-onset patients.

Finally, several genome wide association (GWA) studies have identified a number of SNPs significantly associated to PD [Fung et al., 2006; Gao et al., 2009; Latourelle et al., 2009; Pankratz et al., 2009]. Two recent reports confirmed the association to known genes (i.e. *SNCA* and *MAPT*), and identified two new risk regions on 1q32 and 4p15 [Satake et al., 2009; Simón-Sánchez et al., 2009]. Consistent with our results, none of these GWA studies identified the regions containing the three miRNA-133 and the *PITX3* genes associated with PD.

In conclusion, we report a search for DNA variants in the miRNA-133 and *PITX3* genes in PD patients. We did not find significant associations between these genes variants and PD-risk. Overall, our data indicate that mutations/polymorphisms in these genes involved in dopaminergic neurons survival did not contribute to the risk of suffering PD.

**ACKNOWLEDGEMENTS.** This work was supported by grants from the Spanish Fondo de Investigaciones Sanitarias-Fondos FEDER European Union (FIS-05/008 and 08/0915). LDM is a predoctoral fellow of FICYT-Principado de Asturias. AIC is the recipient of a Contrato de Apoyo a la Investigación- Fondo de Investigaciones Sanitarias.

### **Conflicts of interest.**

The authors declare they did not have conflicts of interest.

### **Disclosure statement.**

The authors, disclose any actual or potential conflicts of interest (such as employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/ registrations, and grants or other funding), including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) their work.



**Table 1.** Main characteristics of patients with PD and controls from the populations of Asturias and Navarra. Patients with a family history of PD were those with at least one or second degree relative also diagnosed with PD. Patients with an age at the onset of symptoms  $\leq 50$  years had an early onset PD (see [http://www.ninds.nih.gov/disorders/parkinsons\\_disease](http://www.ninds.nih.gov/disorders/parkinsons_disease) for the diagnostic criteria and the definition of early and late-onset PD). Patients with a positive family history of PD had at least one first-degree relative also affected with PD. The frequencies of carriers of the common *LRRK2* mutations are also indicated.

	Patients Asturias (n=777)	Controls Asturias (n=650)	Patients Navarra (n=250)	Controls Navarra (n=210)
Mean age (years)	58 $\pm$ 13	58 $\pm$ 18	57 $\pm$ 12	72 $\pm$ 9
Age range (years)	16-91	18-96	17-84	37-98
Male	52 %	54 %	62%	46%
Early onset PD ( $\leq 50$ years)	181 (23 %)	-	71 (28%)	-
Family history of PD	225 (29 %)	-	85 (34%)	-
<i>LRRK2 R1441G/C</i>	19 (2%)	-	7 (2%)	
<i>LRRK2 G2019S</i>	18 (2%)	-	9 (4%)	

**Table 2.** Genotype frequencies for the three miR-133 polymorphisms in patients and controls. Patients with a family history of PD were those with at least one or second degree relative also diagnosed with PD. No significant difference for the genotype frequencies was found between younger (<50 years) and older controls.

	PD (n=575)	Controls (n=650)
miR-133a-1:+85 A/C#		
AA	0	0
AC	29 (5%)	29 (4%)
CC	546 (95%)	621 (96%)
miR-133a-2: -191 A/G		
AA	29 (5%)	33 (5%)
AG	178 (31%)	218 (34%)
GG	368 (64%)	399 (61%)
miR133b: -90 ins/delA*		
ins/ins	488 (85%)	587 (90%)
ins/del	81 (14%)	63 (10%)
del/del	6 (1%)	0
<i>PITX3</i> : c. 285 T/C Rs2281983		
TT	215 (37%)	227 (35%)
TC	274 (48%)	315 (48%)
CC	86 (15%)	108 (17%)

\* p=0.03 (Yate's chi-squared), del/del PD vs. Total controls.

#The nucleotide variants were numbered according to the mature miRNA sequences, with -1 as the first nucleotide 5' upstream and +1 as the first nucleotide 3' downstream.

**Table 3.** Genotype frequencies for the 133b polymorphism in patients and controls from total PD patients and controls, and the two Spanish populations studied (Asturias and Navarra).

	<b>133b -90 ins/ins</b>	<b>133b -90 ins/del</b>	<b>133b -90 del/del</b>
<b><u>Asturias *</u></b>			
<b>Controls (n=650)</b>	<b>574 (88%)</b>	<b>76 (12%)</b>	<b>0</b>
<b>Patients (n=777)</b>	<b>679 (87%)</b>	<b>91 (12%)</b>	<b>7 (1%)</b>
<b>Mean-onset age</b>	58 ± 13	58 ± 13	55 ± 12
<b>Early-onset PD (n=181)</b>	<b>154 (85%)</b>	<b>25 (14%)</b>	<b>2 (1%)</b>
<b><u>Navarra</u></b>			
<b>Controls (n=210)</b>	<b>187 (89%)</b>	<b>21 (10%)</b>	<b>2 (1%)</b>
<b>Patients (n=250)</b>	<b>226 (90%)</b>	<b>23 (9%)</b>	<b>1 (0.4%)</b>
<b>Mean-onset age</b>	57 ± 12	57 ± 16	<b>45 and 58</b>
<b>Early-onset PD (n=71)</b>	<b>62 (87%)</b>	<b>8 (12%)</b>	<b>1 (1%)</b>
<b><u>Total #</u></b>			
<b>Controls (n=860)</b>	<b>761 (88%)</b>	<b>97 (11%)</b>	<b>2 (&lt;1%)</b>
<b>Patients (n=1,027)</b>	<b>905 (88%)</b>	<b>114 (11%)</b>	<b>8 (&lt;1%)</b>
<b>Mean-onset age</b>	58 ± 12	57 ± 13	55 ± 13
<b>Early-onset PD (n= 252)</b>	<b>216 (86%)</b>	<b>33 (13%)</b>	<b>3 (1%)</b>
<b><i>LRRK2</i> R1441G/C G2019S (n=53)</b>	<b>49 (92%)</b>	<b>3 (6%)</b>	<b>1 (2%)</b>
<b>No <i>LRRK2</i> mutation (n=974)</b>	<b>856 (88%)</b>	<b>111 (11%)</b>	<b>7 (1%)</b>

# The two delA/delA patients from Navarra had onset-ages of 45 and 58 years.

\* p=0.01 (p=0.04, Yate's)

# Total: p=0.03 (p=0.07, Yate's)

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***Supplementary Table.***

Sequences of the primers to amplify the miRNA-133b, 133a-1, 133a-2, and *PITX3* genes, and size of the PCR fragments and annealing temperatures. The 3' UTR of *PITX3* was amplified in two overlapping fragments.

<b>amplified region</b>	<b>Primers forward, F / reverse, R</b>	<b>Size Base pairs</b>	<b>Annealing temperature</b>
<b><i>PITX3</i> exon 2</b>	F: TAAGGAGCCCCACGCAGC	419 bp	60 °C
	R: GGTCACAGAGCGCCCAAG		
<b><i>PITX3</i> exon 3</b>	F: CAGCGAGTGGCTTAGGAGGT	463 bp	60 °C
	R: GGAGCAGAGGCTGGAGGT		
<b><i>PITX3</i> exon 4</b>	F: CCCTTCAGCCGCTGGGAC	443 bp	60 °C
	R: GTCCTCCGGGGCCGTGT		
<b><i>PITX3</i> 3'UTR</b>	F: TCGCAGCCCGTCTTCTCG	449 bp	60 °C
	R: GCAAGCCAGTCAAAATGACC		
<b><i>PITX3</i> 3'UTR</b>	F: TGGAAAGGCCCGTATGAGC	438 bp	60 °C
	R: GTACAGGAACGAAGTGAGGG		
<b>miRNA 133a-1</b>	F: CAGTGACTGAAGCATTGGTATGATAA R: GCTTTTTATAAACATCTTTAACCATTC	357 bp	58 °C
<b>miRNA 133a-2</b>	F: GCAGAGCGAGAGGGTGGT R: GCTTCACTTACTTGGAGCTGAC	444 bp	59 °C
<b>miRNA 133b</b>	F: GCATTTGACAGATGCTAATGCT R: GCATAAGAATATGGTGAAAAAACC	306 bp	56 °C