

***FGF20* rs12720208 SNP and microRNA-433 variation: no association with Parkinson's disease in Spanish patients**

Lorena de Mena¹, Lucía F. Cardo¹, Eliecer Coto^{1,3}, Ana Miar¹, Marta Díaz¹, Ana I. Corao¹, Belén Alonso¹, René Ribacoba², Carlos Salvador², Manuel Menéndez², Germán Morís², and Victoria Alvarez¹.

¹Genética Molecular, Hospital Universitario Central de Asturias, Oviedo, Spain.

²Neurología, Hospitales Universitario Central Asturias, San Agustín-Avilés, and Alvarez Buylla-Mieres, Asturias, Spain.

³ Departamento de Medicina, Universidad de Oviedo, Oviedo, Spain.

Correspondence:

Dra. Victoria Alvarez
Genética Molecular
Hospital Central Asturias-Maternidad
33006 – Oviedo – SPAIN
Tel. (34).985.10.79.68
Email: victoria.alvarez@sespa.princast.es

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

Running short title:

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., A.I.C., B.A., and A.M. performed all the experiments. G.M., R.R., C.S., G.M., and M.M. recruited the patients and obtained all the clinical and anthropometric data.

Abstract.

DNA variation at the *FGF20* gene has been associated with Parkinson's disease (PD). In particular, SNP rs12720208 in the 3' untranslated region (3'UTR) was linked to PD-risk through a mechanism that would implicate a differential binding to micro RNA – 433 (miR-433). The reduction of the affinity of miR-433 to the 3'UTR would result in increased *FGF20* expression and upregulation of alpha-synuclein, that could in turn promote dopaminergic neurons degeneration. We genotyped the rs12720208 SNP in a total of 512 PD patients and 258 healthy controls from Spain, and searched for miR-433 variants in the patients. We did not find significant differences in allele and genotype frequencies between patients and controls. None of the patients had miR-433 variants. In conclusion, our work did not confirm the association between rs12720208 and PD, or an effect of miR-433 variants on this disease.

Text:

Parkinson disease (PD) is a neurodegenerative disorder that affects 2% of the people > 65 years [4, 6]. PD is characterized by a loss of dopaminergic neurons (DNs) within the *substantia nigra* and both, acquired and inherited risk factors have been implicated in DN-death [13, 28]. The existence of affected relatives is a risk factor to develop PD, and linkage analysis in families with several affected identified at least 16 loci/genes implicated in PD. Most of the PD patients are sporadic cases, and the genetic predisposition resides in the variation (polymorphisms) at several candidate genes. A complete genome screening identified a chromosome 8 region positively associated with PD [25]. Further studies reported a significant association to single nucleotide polymorphisms (SNPs) in the Fibroblast Growth Factor 20 (*FGF20*) gene, with the strongest risk linked to allele T of SNP rs12720208 in the 3' untranslated region (3' UTR) [30, 31] (see the PDgene for a summary of the genetic association studies, www.PDgene.org).

FGF20 is a growth factor expressed in DNs [10, 15, 21]. FGF2, another member of the FGF family, upregulated the expression of α -synuclein in cultured rat ventral midbrain DN [22]. Alpha-synuclein is the main component of the Lewy bodies, the pathological hallmark of PD [27]. Mutations in the α -synuclein gene (*SNCA*), including gene duplications, caused familial autosomal-dominant PD [14, 26, 34]. In addition, overexpression of α -synuclein increased the vulnerability of DNs to neurotoxins and promoted cell death [24, 35]. As FGF2 and FGF20 binds

to the same receptor (FGFR1), FGF20 could also affect α -synuclein levels in DNs. This could explain the association of *FGF20* variants with PD-risk.

SNP rs12720208 is in the 3' UTR of *FGF20*, in a sequence that binds to microRNA (miRNA) 433. MiRNAs are small (approximately 20 nucleotides long) non-coding RNAs that bind to the 3' UTR of mRNAs and negatively regulate translation. The miRNA genes encode a large RNA transcript, the pri-miRNA, that are first processed to the approximately 90 nucleotides long 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 [1]. Through this process, miRNAs regulate cellular processes such as differentiation, growth, proliferation, and apoptosis [29]. Recent reports showed that the deregulation of some miRNAs could result in DNs death and PD [11, 17].

To test the effect of SNP rs12720208 on PD-risk in our population we genotyped 512 (mean age at diagnosis 58 ± 13 years, range 16-91 years; 45% females; 33% with at least one relative also affected by PD) and 258 healthy controls (mean age 73 ± 11 , range 24- 97; 62% females). Patients with an onset of symptoms at an age ≤ 50 years were classified as early-onset PD (see http://www.ninds.nih.gov/disorders/parkinsons_disease for the definition of early and late-onset PD). Patients were recruited through the Movement Disorders Unit of Hospital Universitario Central Asturias (HUCA), and controls were Blood Bank donors or healthy spouses of patients. Patients and controls were Caucasians and from the same region (Asturias, Northern Spain, total population 1 million), and gave their informed consent to participate in the study, approved by the Ethical Committee of HUCA.

SNP rs12720208 was genotyped through polymerase chain reaction amplification with primers CTTTCTTGTCATAGTTCCCATC (forward) and CATTCTTCAACATGAAGTCTC (reverse; annealing at 58°C; fragment size, 334 bp), followed by digestion with the restriction enzyme *Hin1II*. Digestions were electrophoresed on 3% agarose gels to visualize the bands of 252bp + 82 bp (allele T) and 141bp +111 bp+82 bp (allele C). To determine the accuracy of this genotyping method, we sequenced the PCR fragment and confirmed the genotype of all the TT (n=2), and 20 TC and 20 CC individuals. In all the patients, we also sequenced exons 31 and 41 of the *LRRK2* gene to define the two common mutations R1441G and G2019S [18]. Allele and genotype frequencies in patients and controls were compared through a Chi-squared test. This test was also

used to determine whether the genotype frequencies deviated from the Hardy-Weinberg equilibrium. The ANOVA was used to compare the mean age between the groups. Logistic regression was used to determine the association of allele/genotypes after correcting by age and gender. All statistical analyses were performed with the SPSS statistical package (v.11.0) and a $p < 0.05$ was considered as statistically significant.

The rs12720208 genotype frequencies did not differ from those expected under the Hardy-Weinberg equilibrium in patients and controls. Allele and genotype frequencies did not differ between total patients and controls ($p=0.9$), and we did not find significant differences in mean onset age between the genotypes in the patients (**Table 1**). After correcting by age and gender, the association of rs12720208 to PD remained non significant ($p=0.89$). We compared the frequencies between patients with/without *LRRK2* mutation, or with/without affected relatives, and no significant differences were found between the groups (**Table 2**). We did not find significant differences between patients with early onset-PD (≤ 50 years; $n=127$) and late onset-PD (>50 years; $n=385$) (data not shown). Together, our data indicated that the *FGF20* SNP rs12720208 did not contribute to the risk of developing PD in our population. This was in agreement with previous works that reported a lack of association to various *FGF20* variants [3, 7, 8, 23, 32].

DNA variants that create or destroy miRNA target sites could result in phenotypic variation and this could explain the association of some 3' UTR polymorphisms to diseases [9]. To date, more than 600 human miRNAs have been characterized (see www.microna.sanger.ac.uk). In addition to *FGF20*, other genes that have been implicated in DN survival and PD (i.e. the brain-derived neurotrophic factor, *BDNF*) contain miR-433 target sequences in their 3'UTRs (see the miR-Ontology database at <http://ferrolab.dmi.unict.it/miro> for a summary of the genes, diseases and biological functions/processes regulated by miR-433). Changes in the levels and activities of BDNF have been described in a number of neurodegenerative disorders, including Alzheimer disease and Parkinson disease [20, 36]. In addition, BDNF gene variants were related with the risk of developing PD [12, 16, 33]. In the case of SNP rs12720208, allele T would disrupt the binding to miR-433, increasing the stability and translation of *FGF20* [31]. DNA variants in miRNA genes could also result in a higher risk for diseases. For instance, nucleotide changes in mature miRNAs that increase or reduce the binding to a 3' UTR would result in reduced or increased gene translation [5, 19].

To determine whether miR-433 variants were associated with PD we searched for nucleotide changes through direct sequencing and Denaturing High Performance Liquid Chromatography (dHPLC). First, a 471 bp DNA-fragment was PCR-amplified from 50 patients with primers that flanked the whole pre-miRNA sequence (reference sequence ENSG00000207569; www.ebi.ac.uk/Ensembl): GCCGTAGGATCTGTTCATTG (forward) and TCCAAATCGACGACCAAAC (reverse; annealing 60°C) and both strands were sequenced using BigDye chemistry (www.appliedbiosystems.com). These patients were negative for the two *LRRK2* mutations, and 25 had affected relatives while 25 were apparently sporadic cases. We did not find nucleotide changes in these patients. We then amplified the DNA of 400 patients (randomly chosen from the total 512 patients) and searched for nucleotide changes through dHPLC in a *Varian Helix System* with a linear binary gradient created with buffers Varian Helix A (triethylammonium amine, TEAA) and B (TEAA + 25% acetonitrile) (www.varianinc.org). All the samples showed a single elution peak, indicating the absence of nucleotide changes in all these patients. Because dHPLC is an indirect technique for the detection of DNA variation, we cannot exclude that some miR-433 DNA variants were not detected. However, the rate for false negatives should be very low (<2% according to most authors), and this suggested that miR-433 DNA changes should be rarely (at the best) linked to PD-risk [2].

In conclusion, the common *FGF20* rs12720208 SNP was not associated with the risk for PD in our population. In addition, we did not find nucleotide changes in miR-433 (that binds to the 3' UTR *FGF20* mRNA) among our PD patients.

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.

Table 1. *FGF20* rs12720208 allele and genotype frequencies in PD patients and healthy controls, and mean age in controls and mean onset age in patients in the different genotypes.

	Controls N=258	Mean age 73±11 years	Patients N=512	Mean onset age 58±13 years
Genotypes				
TT	0	-	2 (0.4%)	58.5 ± 16.2
TC	44 (17.1%)	73.3 ± 8.9	84 (16.4%)	57.6 ± 13.3
CC	214 (82.9%)	72.5 ± 11.1	426 (83.2%)	57.8 ± 13.5
Alleles				
T	44 (8.5%)		88 (9.5%)	
C	472 (95.5%)		936 (90.5%)	

Table 2. *FGF20* rs12720208 frequencies in the patients with/without affected relatives, and with/without *LRRK2* mutation.

	Patients without affected relatives (mean onset age) N= 344 (57.9 ± 13.6)	Patients with affected relatives (mean onset age) N=168 (57.8 ± 13)	Patients LRRK2 + (mean onset age) N=21 (57 ± 9)	Patients LRRK2 – (mean onset age) N= 491 (57.9 ± 13.5)
Genotypes				
TT	2 (<1%) 58.5 ± 16.2	0	1 (<1%) 47	1 (<1%) 70
TC	54 (16%) 57.8 ± 14.3	30 (18%) 57.3 ± 11.7	3 (18%) 66.5 ± 12	81 (17%) 57.3 ± 13.3
CC	288 (84%) 57.7 ± 13.6	138 (82%) 57.8 ± 13.3	17 (81.5%) 56.4 ± 8.4	409 (83%) 57.9 ± 13.6
Alleles				
T	58 (8.4%)	30 (8.9%)	5 (9.6%)	83 (8.4%)
C	630 (91.6%)	306 (91.1%)	37 (90.4%)	899 (91.6%)

References.

- [1] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell* 116 (2004) 281-297.
- [2] I.M. Buyse, P. Fang, K.T. Hoon, R.E. Amir, H.Y. Zoghbi, B.B. Roa, Diagnostic testing for Rett syndrome by DHPLC and direct sequencing analysis of the MECP2 gene: identification of several novel mutations and polymorphisms, *American journal of human genetics* 67 (2000) 1428-1436.
- [3] J. Clarimon, G. Xiromerisiou, J. Eerola, V. Gournali, O. Hellstrom, E. Dardiotis, T. Peuralinna, A. Papadimitriou, G.M. Hadjigeorgiou, P.J. Tienari, A.B. Singleton, Lack of evidence for a genetic association between FGF20 and Parkinson's disease in Finnish and Greek patients, *BMC Neurol* 5 (2005) 11.
- [4] M.C. de Rijk, L.J. Launer, K. Berger, M.M. Breteler, J.F. Dartigues, M. Baldereschi, L. Fratiglioni, A. Lobo, J. Martinez-Lage, C. Trenkwalder, A. Hofman, Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group, *Neurology* 54 (2000) S21-23.
- [5] R. Duan, C. Pak, P. Jin, Single nucleotide polymorphism associated with mature miR-125a alters the processing of pri-miRNA, *Hum Mol Genet* 16 (2007) 1124-1131.
- [6] S. Fahn, Description of Parkinson's disease as a clinical syndrome, *Ann N Y Acad Sci* 991 (2003) 1-14.
- [7] H.C. Fung, S. Scholz, M. Matarin, J. Simon-Sanchez, D. Hernandez, A. Britton, J.R. Gibbs, C. Langefeld, M.L. Stiebert, J. Schymick, M.S. Okun, R.J. Mandel, H.H. Fernandez, K.D. Foote, R.L. Rodriguez, E. Peckham, F.W. De Vrieze, K. Gwinn-Hardy, J.A. Hardy, A. Singleton, Genome-wide genotyping in Parkinson's disease and neurologically normal controls: first stage analysis and public release of data, *Lancet Neurol* 5 (2006) 911-916.
- [8] X. Gao, W.K. Scott, G. Wang, G. Mayhew, Y.J. Li, J.M. Vance, E.R. Martin, Gene-gene interaction between FGF20 and MAOB in Parkinson disease, *Ann Hum Genet* 72 (2008) 157-162.
- [9] M. Georges, W. Coppieters, C. Charlier, Polymorphic miRNA-mediated gene regulation: contribution to phenotypic variation and disease, *Curr Opin Genet Dev* 17 (2007) 166-176.
- [10] M.A. Hauser, Y.J. Li, S. Takeuchi, R. Walters, M. Noureddine, M. Maready, T. Darden, C. Hulette, E. Martin, E. Hauser, H. Xu, D. Schmechel, J.E. Stenger, F. Dietrich, J. Vance, Genomic convergence: identifying candidate genes for Parkinson's disease by combining serial analysis of gene expression and genetic linkage, *Hum Mol Genet* 12 (2003) 671-677.
- [11] S.S. Hebert, B. De Strooper, Molecular biology. miRNAs in neurodegeneration, *Science (New York, N.Y)* 317 (2007) 1179-1180.
- [12] C.J. Hong, H.C. Liu, T.Y. Liu, C.H. Lin, C.Y. Cheng, S.J. Tsai, Brain-derived neurotrophic factor (BDNF) Val66Met polymorphisms in Parkinson's disease and age of onset, *Neurosci Lett* 353 (2003) 75-77.
- [13] Y. Huang, L. Cheung, D. Rowe, G. Halliday, Genetic contributions to Parkinson's disease, *Brain Res Brain Res Rev* 46 (2004) 44-70.

- [14] P. Ibanez, A.M. Bonnet, B. Debarges, E. Lohmann, F. Tison, P. Pollak, Y. Agid, A. Durr, A. Brice, Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease, *Lancet* 364 (2004) 1169-1171.
- [15] M. Jeffers, R. Shimkets, S. Prayaga, F. Boldog, M. Yang, C. Burgess, E. Fernandes, B. Rittman, J. Shimkets, W.J. LaRochelle, H.S. Lichenstein, Identification of a novel human fibroblast growth factor and characterization of its role in oncogenesis, *Cancer Res* 61 (2001) 3131-3138.
- [16] S. Karamohamed, J.C. Latourelle, B.A. Racette, J.S. Perlmutter, G.F. Wooten, M. Lew, C. Klein, H. Shill, L.I. Golbe, M.H. Mark, M. Guttman, G. Nicholson, J.B. Wilk, M. Saint-Hilaire, A.L. DeStefano, R. Prakash, S. Tobin, J. Williamson, O. Suchowersky, N. Labell, B.N. Growdon, C. Singer, R. Watts, S. Goldwurm, G. Pezzoli, K.B. Baker, M.L. Giroux, P.P. Pramstaller, D.J. Burn, P. Chinnery, S. Sherman, P. Vieregge, I. Litvan, J.F. Gusella, R.H. Myers, A. Parsian, BDNF genetic variants are associated with onset age of familial Parkinson disease: GenePD Study, *Neurology* 65 (2005) 1823-1825.
- [17] K.S. Kosik, The neuronal microRNA system, *Nature reviews* 7 (2006) 911-920.
- [18] I.F. Mata, O.A. Ross, J. Kachergus, C. Huerta, R. Ribacoba, G. Moris, M. Blazquez, L.M. Guisasola, C. Salvador, C. Martinez, M. Farrer, V. Alvarez, LRRK2 mutations are a common cause of Parkinson's disease in Spain, *Eur J Neurol* 13 (2006) 391-394.
- [19] A. Mencia, S. Modamio-Hoybjor, N. Redshaw, M. Morin, F. Mayo-Merino, L. Olavarrieta, L.A. Aguirre, I. del Castillo, K.P. Steel, T. Dalmay, F. Moreno, M.A. Moreno-Pelayo, Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss, *Nat Genet* 41 (2009) 609-613.
- [20] M. Mogi, A. Togari, T. Kondo, Y. Mizuno, O. Komure, S. Kuno, H. Ichinose, T. Nagatsu, Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease, *Neurosci Lett* 270 (1999) 45-48.
- [21] S. Ohmachi, Y. Watanabe, T. Mikami, N. Kusu, T. Ibi, A. Akaike, N. Itoh, FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain, *Biochem Biophys Res Commun* 277 (2000) 355-360.
- [22] H.J. Rideout, P. Dietrich, M. Savalle, W.T. Dauer, L. Stefanis, Regulation of alpha-synuclein by bFGF in cultured ventral midbrain dopaminergic neurons, *J Neurochem* 84 (2003) 803-813.
- [23] W. Satake, I. Mizuta, S. Suzuki, Y. Nakabayashi, C. Ito, M. Watanabe, A. Takeda, K. Hasegawa, S. Sakoda, M. Yamamoto, N. Hattori, M. Murata, T. Toda, Fibroblast growth factor 20 gene and Parkinson's disease in the Japanese population, *Neuroreport* 18 (2007) 937-940.
- [24] B.L. Schneider, C.R. Seehus, E.E. Capowski, P. Aebischer, S.C. Zhang, C.N. Svendsen, Over-expression of alpha-synuclein in human neural progenitors leads to specific changes in fate and differentiation, *Hum Mol Genet* 16 (2007) 651-666.
- [25] W.K. Scott, M.A. Nance, R.L. Watts, J.P. Hubble, W.C. Koller, K. Lyons, R. Pahwa, M.B. Stern, A. Colcher, B.C. Hiner, J. Jankovic, W.G. Ondo, F.H. Allen, Jr., C.G. Goetz, G.W. Small, D. Masterman, F. Mastaglia, N.G. Laing, J.M. Stajich,

- B. Slotterbeck, M.W. Booze, R.C. Ribble, E. Rampersaud, S.G. West, R.A. Gibson, L.T. Middleton, A.D. Roses, J.L. Haines, B.L. Scott, J.M. Vance, M.A. Pericak-Vance, Complete genomic screen in Parkinson disease: evidence for multiple genes, *JAMA* 286 (2001) 2239-2244.
- [26] A.B. Singleton, M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M.R. Cookson, M. Muentert, M. Baptista, D. Miller, J. Blancato, J. Hardy, K. Gwinn-Hardy, alpha-Synuclein locus triplication causes Parkinson's disease, *Science* 302 (2003) 841.
- [27] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, Alpha-synuclein in Lewy bodies, *Nature* 388 (1997) 839-840.
- [28] K. Steece-Collier, E. Maries, J.H. Kordower, Etiology of Parkinson's disease: Genetics and environment revisited, *Proc Natl Acad Sci U S A* 99 (2002) 13972-13974.
- [29] M.A. Valencia-Sanchez, J. Liu, G.J. Hannon, R. Parker, Control of translation and mRNA degradation by miRNAs and siRNAs, *Genes Dev* 20 (2006) 515-524.
- [30] J.M. van der Walt, M.A. Nouredine, R. Kittappa, M.A. Hauser, W.K. Scott, R. McKay, F. Zhang, J.M. Stajich, K. Fujiwara, B.L. Scott, M.A. Pericak-Vance, J.M. Vance, E.R. Martin, Fibroblast growth factor 20 polymorphisms and haplotypes strongly influence risk of Parkinson disease, *Am J Hum Genet* 74 (2004) 1121-1127.
- [31] G. Wang, J.M. van der Walt, G. Mayhew, Y.J. Li, S. Zuchner, W.K. Scott, E.R. Martin, J.M. Vance, Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of alpha-synuclein, *American journal of human genetics* 82 (2008) 283-289.
- [32] C. Wider, J.C. Dachselt, A.I. Soto, M.G. Heckman, N.N. Diehl, M. Yue, S. Lincoln, J.O. Aasly, K. Haugarvoll, J.Q. Trojanowski, S. Papapetropoulos, D. Mash, A. Rajput, A.H. Rajput, J.M. Gibson, T. Lynch, D.W. Dickson, R.J. Uitti, Z.K. Wszolek, M.J. Farrer, O.A. Ross, FGF20 and Parkinson's disease: no evidence of association or pathogenicity via alpha-synuclein expression, *Mov Disord* 24 (2009) 455-459.
- [33] G. Xiomerisiou, G.M. Hadjigeorgiou, J. Eerola, H.H. Fernandez, V. Tsimourtou, R. Mandel, O. Hellstrom, K. Gwinn-Hardy, M.S. Okun, P.J. Tienari, A.B. Singleton, BDNF tagging polymorphisms and haplotype analysis in sporadic Parkinson's disease in diverse ethnic groups, *Neurosci Lett* 415 (2007) 59-63.
- [34] J.J. Zarranz, J. Alegre, J.C. Gomez-Esteban, E. Lezcano, R. Ros, I. Ampuero, L. Vidal, J. Hoenicka, O. Rodriguez, B. Atares, V. Llorens, E. Gomez Tortosa, T. del Ser, D.G. Munoz, J.G. de Yébenes, The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia, *Ann Neurol* 55 (2004) 164-173.
- [35] W. Zhou, J. Schaack, W.M. Zawada, C.R. Freed, Overexpression of human alpha-synuclein causes dopamine neuron death in primary human mesencephalic culture, *Brain Res* 926 (2002) 42-50.
- [36] C. Zuccato, E. Cattaneo, Brain-derived neurotrophic factor in neurodegenerative diseases, *Nat Rev Neurol* 5 (2009) 311-322.