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

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**Running short title:**

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**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 locigenes 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 $\alpha$-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 $\leq$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 CTTTCTTGTCAATGTTCCATC (forward) and CATTTCTTCAACATGAAGTCTC (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.microrna.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.ensembl.org): GCCGTAGGATCTGTTCATTG (forward) and TCCAAATCGACGACCAAAC (reverse; annealing 60ºC) 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 dDHPLC 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.

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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.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Controls N=258</th>
<th>Mean age 73±11 years</th>
<th>Patients N=512</th>
<th>Mean onset age 58±13 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>0</td>
<td>-</td>
<td>2 (0.4%)</td>
<td>58.5 ± 16.2</td>
</tr>
<tr>
<td>TC</td>
<td>44 (17.1%)</td>
<td>73.3 ± 8.9</td>
<td>84 (16.4%)</td>
<td>57.6 ±13.3</td>
</tr>
<tr>
<td>CC</td>
<td>214 (82.9%)</td>
<td>72.5 ± 11.1</td>
<td>426 (83.2%)</td>
<td>57.8 ±13.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Controls N=258</th>
<th>Patients N=512</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>44 (8.5%)</td>
<td>88 (9.5%)</td>
</tr>
<tr>
<td>C</td>
<td>472 (95.5%)</td>
<td>936 (90.5%)</td>
</tr>
<tr>
<td>Genotypes</td>
<td>Patients without affected relatives (mean onset age)</td>
<td>Patients with affected relatives (mean onset age)</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>N= 344</td>
<td>N= 168</td>
</tr>
<tr>
<td></td>
<td>(57.9 ± 13.6)</td>
<td>(57.8 ± 13)</td>
</tr>
<tr>
<td>TT</td>
<td>2 (&lt;1%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>58.5 ± 16.2</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>54 (16%)</td>
<td>30 (18%)</td>
</tr>
<tr>
<td></td>
<td>57.8 ± 14.3</td>
<td>57.3 ± 11.7</td>
</tr>
<tr>
<td>CC</td>
<td>288 (84%)</td>
<td>138 (82%)</td>
</tr>
<tr>
<td></td>
<td>57.7 ± 13.6</td>
<td>57.8 ± 13.3</td>
</tr>
<tr>
<td>Alleles</td>
<td>T 58 (8.4%)</td>
<td>30 (8.9%)</td>
</tr>
<tr>
<td></td>
<td>C 630 (91.6%)</td>
<td>306 (91.1%)</td>
</tr>
</tbody>
</table>
References.


