Neuroscience Letters 675 (2018) 68–73

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Evaluation of *FGF 20* variants for susceptibility to Parkinson's disease in Eastern Indians

Dipanwita Sadhukhan[a], Gautami Das[a], Arindam Biswas[a], Soumitra Ghosh[a], Shyamal K. Das[b], Kunal Ray[c], Jharna Ray[a,*]

[a] S.N. Pradhan Centre for Neurosciences, University of Calcutta, Kolkata, India
[b] Movement Disorders Clinic, Bangur Institute of Neurosciences, Kolkata, India
[c] Academy of Scientific & Innovative Research, New Delhi, India

ARTICLE INFO

Keywords:
Parkinson's disease
Fibroblast growth factor 20
Single nucleotide polymorphism
Association
Mi*R*-3189-3p

ABSTRACT

Background: Parkinson's disease (PD) is the second most common neurodegenerative disease and has a complex etiology. Single nucleotide polymorphisms in the 3′-untranslated region of *Fibroblast growth factor 20* (*FGF 20*) have been reported to be associated with PD; however, the results are controversial. Although FGF20 enhances the survival of dopaminergic neurons, it may also result in PD susceptibility by altering alpha-synuclein expression.

Materials and methods: To identify and characterize genetic risk variants in *FGF* 20 in Eastern Indian PD patients, 2 SNPs of *FGF 20* (rs1721100 and rs2720208) were genotyped in 336 PD cases and 313 ethnically matched controls by PCR-RFLP.

Results: We observed statistically significant differences in genotypic and allelic frequencies of rs1721100 between PD cases and controls but not for rs12720208. Haplotype G-C showed a significant protective effect against PD. A functional assay revealed that the risk allele C at rs1721100 has little or no effect on relative luciferase activity from a reporter construct in the presence of mi*R*-3189-3p, whereas allele G results in significant dose-dependent reduction.

Conclusion: Our results suggest that *FGF 20* is a susceptibility gene for PD in Eastern Indians.

1. Introduction

Parkinson's disease (PD) (OMIM #168600) is the second most common progressive neurodegenerative disorder and results from selective degeneration of dopaminergic neurons in the substantia nigra (SN) of the midbrain. The accumulation of proteinaceous aggregates, Lewy bodies (LBs), is a pathological hallmark of a PD affected brain. Apart from mutations in candidate genes, interaction between susceptibility genes and environmental factors play a significant role in the complex etiology of the disease. *Fibroblast Growth Factor 20* (*FGF 20;* [MIM*605558]), encoding a neurotrophic growth factor, is known to enhance the survival of dopaminergic neurons [1]. By binding to FGF receptor 1c (FGFR1c), which is highly expressed in SN, FGF20 promotes differentiation of neural stem cells into tyrosine-hydroxylase-positive cells [1,2]. FGF20 has also been shown to regulate the expression of alpha-synuclein (SNCA) [3], a candidate gene for PD. Association studies on single nucleotide polymorphisms (SNPs) in the 3′-untranslated region of *FGF 20* (i.e. rs1721100 and rs12720208) with PD across different ethnic populations show contradictory results [3–7]. Herein, we determined the role of these two SNPs in PD patients from Eastern India by case-controlled study followed by functional validation.

2. Materials and methods

2.1. Recruitment of study subjects

Patients were diagnosed at the Movement Disorders Clinic, Bangur Institute of Neurosciences (BIN), Kolkata, India. A total of 336 unrelated PD patients [male (n = 244), female (n = 92); mean age at onset, 47.42 ± 12.99 years; age at onset ≤ 45 years (n = 140), age at onset > 45 years (n = 196)] and 313 unrelated controls [(male (n = 238); female (n = 75); mean age, 48.3 ± 8.2 years; age ≤ 45 years (n = 131), age > 45 years (n = 182)] with no personal or family history of any neurological symptoms were recruited in the study. All subjects provided written consent for the study as per the guidelines of the Indian Council of Medical Research (ICMR).

* Corresponding author at: S.N. Pradhan Centre for Neurosciences, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, 700 019, India.
E-mail address: jharnaray@gmail.com (J. Ray).

https://doi.org/10.1016/j.neulet.2018.03.059
Received 22 December 2017; Received in revised form 22 March 2018; Accepted 27 March 2018
Available online 28 March 2018

2.2. DNA extraction and genotyping of SNPs

Genomic DNA was prepared from fresh whole blood by a conventional salting out method using sodium perchlorate followed by isopropanol precipitation [8] and then dissolved in TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). Polymerase chain reaction (PCR) was carried out to amplify rs1721100 and rs12720208 using the following two primer pairs respectively:

FGF 20_3F:5′-GAGTTGAAACTTGTAAGGAAATTA-3′, FGF 20_3Rseq1: 5′-TCTCAATATTCTTTCCAAATCCAG-3′, and Fgf G > A F: 5′-CAACCATGTGAGGGTTCCCATT-3′, Fgf G > A R: 5′-TGACCCAAGG AGACGTTCAAAA-3′

to generate 464 bp and 225 bp amplicons, respectively. Genotypes for rs1721100 were determined by restriction digestion of its PCR products with *Bsa*JI (New England Biolabs, UK) (fragment sizes for G-allele: 396 and 68 bp) whereas PagI (Fermentas UAB, Vilnius, Lithuania) was used to identify the genotypes of rs12720208 (fragment sizes for C-allele: 113 and 112 bp). The digested products were separated by 6% polyacrylamide gel electrophoresis, and visualized by ethidium bromide staining. About 10% of the samples were randomly selected for sequencing to rule out genotyping errors.

2.3. Statistical analysis

Hardy-Weinberg equilibrium (HWE) at the polymorphic sites was tested using a chi square test with one degree of freedom. For association study the data was evaluated for *p*-value, odds ratio and 95% confidence interval (CI) using Javastat (http://statpages.info/ctab2x2.html). FDR (false discovery rate) correction was done for multiple testing.

2.4. Haplotype analysis

Haploview (version 4.2) was used to determine the linkage disequilibrium (LD) of the SNPs and the frequency of the different haplotypes formed by the 2 SNPs.

2.5. Human FGF 20 3′UTR luciferase reporter constructs

The *FGF 20* 3′UTR containing the G-allele of rs1721100 was amplified from genomic DNA of a patient homozygous (GG) for the SNP with primer pairs (5′-CGATCGCTCGAGCCACAACCATTCTTTCTTG-3′ and 5′-CCAGCGGCCGCTTGGCTTCAGTCTACTG-3′). The amplified fragments were cloned via TOPO-TA vector-mediated cloning (Invitrogen, CA, USA) into the pSICHECK™-2 dual luciferase vector (Promega, WI, USA) after Not I − Xho I digestion (NEB, MA, USA) as per the manufacturer's protocol. The C-allele of rs1721100 was introduced by site directed mutagenesis (SDM) using Phusion Hot Start II DNA polymerase (Thermo Scientific, Massachusetts, United States) in recombinant reporter vector (pSICHECK™-2G construct) following manufacturer's protocols. The sequences for the SDM primer set used are as follow:

5′-CCCATCATAAAATAATGACCCAAG**C**AGACGTTCAAAATATTAAA GTC-3′ and 5′-GACTTTAATATTTTGAACGTCT**G**CTTGGGTCATTATTTT ATGATGGG-3′.

2.6. Luciferase assay

The SH-SY5Y human neuroblastoma cell line was maintained in DMEM (Dulbecco's Modified Eagle Medium, Gibco, BRL) supplemented with 10% fetal bovine serum, pH 7.4, 5% CO_2 at 37 °C. Approximately 3×10^5 cells were seeded per well in 24-well plates before the day of transfection. Cells were co-transfected with 100 ng of the recombinant pSICHECK™-2 constructs (pSICHECK™-2G and pSICHECK™-2C) containing the *FGF 20* 3′UTR. Different concentrations (0, 2.5, or 10 nM) of hsa-miR3189-3p mimic and 100 nM of hsa-miR3189-3p inhibitor or scrambled miR (10 nM) were used for the various experiments. Cells were harvested 48 h post-transfection with 75 µl of 1X passive lysis buffer. Renilla luciferase activities in cell lysate were measured with the Dual Luciferase assay system (Promega, Madison, WI) in a Glomax 20/20 luminometer (Promega, Madison, WI). The Renilla luciferase values were normalized with corresponding firefly luciferase values, and expressed in ratios as Relative Luciferase Activity. Results were analysed by an unpaired *t*-test.

3. Results

3.1. Association of FGF 20 SNP and haplotype with PD

The entire 3′UTR of *FGF 20* was screened in 50 individuals to identify the presence of nucleotide variants. Four variants (rs1721100, rs12720208, rs1088125 and rs17550395) were identified. Among those, rs1088125 and rs17550395 showed similar allele and genotype frequency distribution between cases and controls, and were thus excluded for further analysis. Only rs1721100 and rs12720208 showed minor allele frequency differences between cases and controls in the initial dataset. In addition, on the basis of (a) contradictory reports on their association in different ethnic populations (not yet tested in India), (b) conflicting evidence of the functional role of rs12720208 with PD [3,6], and also (c) to explore the genetic association of *rs1721100* with PD patho-mechanisms, the present study aimed to genotype rs1721100 and rs12720208 for their potential association with the disease in extended samples including 336 unrelated PD patients and 313 ethnically matched healthy controls from Eastern India.

Both SNPs were within Hardy-Weinberg equilibrium and were not in linkage disequilibrium (LD) either in patients or control groups (r^2 value = 6). Table 1 shows the allele and genotype frequency of SNPs rs1721100 and rs12720208. For the rs1721100C-allele [OR = 1.311; 95% CI = 1.034-1.66; *p*-value = 0.022] and CC genotype [OR = 1.411; 95% CI = 1.015- 1.96; *p*-value = 0.037] were observed to be associated with risk for PD. The study subjects were then stratified to have a better understanding of its influence on mean age at onset and gender. A significant overrepresentation of the C-allele of rs1721100 in male patients (68.3%) relative to male controls (60.1%) [OR = 1.357; 95% CI = 1.030–1.788; *p*-value = 0.025] and female patients (65%) suggested that this SNP may be a risk factor in male PD cases. However, we did not observe any association of this SNP with mean age at onset (data not shown). In the case of rs12720208, neither allelic nor genotype was found to be associated with the disease.

Because only 2 SNPs were examined in our cohort, our association data was corrected for multiple testing. After doing FDR (false discovery rate) for multiple testing, the significance of allelic association between rs1721100 and PD did withstand multiple testing (*p*-value = 0.022, which is less than the adjusted threshold p value of 0.025 for 2 SNPs) (Table 2).

Haplotypes were next determined based on the genotypes at rs1721100 and rs12720208 for each individual using Haploview (version 4.2) (Table 3). We identified a statistically significant protective effect of the G-C haplotype against PD [OR = 0.732; 95% CI = 0.571–0.939; p value = 0.012].

3.2. In silico analysis predicts a miR-3189-3p binding site in the FGF 20 3′UTR

To identify potential miRNA binding sites overlapping rs1721100 that might regulate *FGF 20* expression, Target Scan_7.1 (http://www.targetscan.org/vert_71/) and miR Search v3.0 (https://www.exiqon.com/miRSearch) were used for in silico prediction. Both prediction algorithms predicted hsa-mi*R*-3189-3p to be a putative target for rs1721100. The 8-mer seed match between hsa-mi*R*-3189-3p and the SNP had a context score of binding of 0.57 when the G-allele is present at rs1721100 it can pair with hsa-mi*R*-3189-3p but not the C-allele.