SUPPLEMENTARY MATERIALS

Material and methodology

Study population

After obtaining ethical approval, individuals diagnosed with Parkinson's disease (PD) according to the UK PD Society Brain Bank clinical diagnostic criteria and assessed in our outpatient Neurology department were included in our study. The exclusion criteria for the study included the presence of diseases associated with autophagy, such as diabetes mellitus, cardiovascular diseases, cancer, fatty liver disease, autoimmune diseases, and kidney disease. Additionally, individuals on PD-related medications or those with an active infection at the time of blood collection were excluded.

Patients' motor and non-motor symptoms were evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS). Control group participants were selected from volunteers whose neurological examinations were normal.

DNA isolation

Genomic DNA from patients and controls was obtained from 500 µl of peripheral blood using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany) according to the kit protocol. The quantity and purity of the extracted DNA samples were determined spectrophotometrically (DeNovix, Wilmington, DE, USA) at A260, as well as by measuring the A260/A230 and A260/A280 ratios.

DNA digestion with methylation-specific restriction enzymes

DNA methylation in eukaryotic cells occurs at cytosines followed by guanine, specifically at the C5 position. These regions are known as methylated CpG dinucleotides.[1] Methylation-specific areas are predominantly found in the promoter regions of genes. When these regions are methylated, they cannot be cut by methylation-specific restriction enzymes (MSREs). Conversely, unmethylated cut DNA cannot be amplified by polymerase chain reaction (PCR). Based on this principle, the isolated DNA samples were digested with MSREs (HpaII and Hin6I; Fermentas, Vilnius, Lithuania) for 24 hours. The efficiency of the digestion reactions was evaluated by running the DNA samples on 1% agarose gels. The number of MSRE recognition sites in each promoter region is provided in the Supplementary Table 1 (in the online-only Data Supplement).

PCR

Cut and uncut DNA from participants were used as templates in PCR reactions, with each sample analyzed in triplicate. All samples were processed using a StepOnePlus™ Real-Time PCR System and the StepOne™ Plus v2.3 software (Applied Biosystems, Foster City, CA, USA). Briefly, each 20 µl PCR reaction contained 150 ng gDNA, 1 × SYBR Green master mix (RealQ Plus 2 × Master Mix Green; Ampliqon, Odense, Denmark), 200 nM forward primer, and 200 nM reverse primer. The PCR cycles were as follows: one cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 sec and 55–61°C (according to the specificity and optimum temperature of each primer, not shown) for 30 sec. Ct values generated by the StepOne™ Plus v2.3 software were analyzed using the Livak method, with uncut DNA from each sample as the reference. PCR products were also analyzed on 3% agarose gels containing 0.5 µg/mL ethidium bromide.

Hypomethylated promoters, which were digested more efficiently by MSREs, resulted in no amplification, and only primers were visible on gels. In contrast, hypermethylated promoter regions, which were not digested by MSREs, were efficiently amplified by PCR, using up the primers, so only the amplified DNA was visible on the gel. When methylation was moderate, both amplified DNA and primers were observed.

Statistical analysis

Before starting the study, a power analysis was conducted, which determined that at least 42 participants should be included for statistical significance. Statistical analysis of the data was performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). The conformity of the data to the normal distribution was assessed using the Shapiro–Wilk test. Data were summarized with median, minimum, and maximum values. The correlation analysis of gene methylation levels and age and UPDRS was performed by Spearman correlation coefficient. The Mann–Whitney U test was used to compare two independent groups. A significance level of 0.05 was used for all analyses.

The outliers are removed and statistical analyse is performed after removal.

REFERENCES

1. Unnikrishnan A, Freeman WM, Jackson J, Wren JD, Porter H, Richardson A. The role of DNA methylation in epigenetics of aging. Pharmacol Ther 2019;195:172-185.