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Analysis of mutations and the association between polymorphisms in the *cerebral dopamine neurotrophic factor* (*CDNF*) gene and Parkinson disease

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ABSTRACT

Neurotrophic factors support the survival of dopaminergic neurons. The cerebral dopamine neurotrophic factor (CDNF) is a novel neurotrophic factor with strong trophic activity on dopaminergic neurons comparable to that of glial cell line-derived neurotrophic factor (GDNF). To investigate whether rare or common variants in *CDNF* are associated with Parkinson disease (PD), we performed mutation analysis of *CDNF* and a genetic association study between *CDNF* polymorphisms and PD. We screened 110 early-onset Parkinson disease (EOPD) patients for *CDNF* mutations. Allelic and genotype frequencies of 3 *CDNF* single nucleotide polymorphisms (SNPs) (rs1901650, rs7094179, and rs11259365) were compared in 215 PD patients and age- and sex-matched controls. We failed to identify any mutations in *CDNF* among the EOPD patient sample population. We observed a trend towards increased risk for PD in patients carrying the C allele of SNP rs7094179 (odds ratio (OR) = 1.27, 95% confidence interval (CI) 0.96–1.67). Patients carrying the C allele were susceptible to PD in both dominant (CC + CA vs. AA; OR = 7.20, 95% CI 0.88–59.1) and recessive (CA + AA vs. CC; OR = 0.64, 95% CI 0.41–0.99) models. Genotype and allele frequencies of SNPs rs1901650 and rs11259365 did not differ between PD patients and controls. Our study suggests that the C allele of an intronic *CDNF* SNP (rs7094179) might be an allele for susceptibility to PD. Further studies with larger sample size are required to confirm our results.

Parkinson disease (PD) is one of the most common neurodegenerative diseases in old age second to Alzheimer disease. It is characterized by tremor, rigidity, bradykinesia, and postural instability. Degeneration of dopaminergic neurons is a pathological hallmark of PD. Although the mechanism of dopaminergic neuronal death is not clearly understood yet, it appears that both genetic and environmental factors are involved. Disease-causing mutations and PD susceptibility genes have been identified as genetic causes. Rare mutations in PD-causing genes (*SNCA*, *PARKIN*, *UCHL1*, *PINK1*, *DJ-1*, *LRRK2*, *ATP13A2*, and *Omi/HTRA2*) that had been previously identified in familial PD were later confirmed in familial or sporadic PD in diverse ethnic groups [21,8,12,1,32,30,23,26]. Some of these genes were identified by linkage analysis in PD families. Additionally, mutations in other genes were found by candidate gene analysis of large PD samples [19,11]. *NR4A2* (*NURR1*), a member of the orphan nuclear receptor superfamily, is highly expressed in the developing and adult ventral midbrain, and is required for the acquisition and maintenance of the dopaminergic phenotype in nigrostriatal neurons [31,6]. Mutations in *NR4A2*, which result in reduced expression of the NR4A2 protein, were reported to be associated with late-onset familial PD [11]. Neurotrophic factors such as GDNF and neurturin (NRTN) not only protect but also rescue and repair adult dopaminergic neurons in adult animal models of PD [29,7]. A study indicates that levels of GDNF are decreased in PD brain [2]. Polymorphisms in RET, the receptor for GDNF was reported not to be associated with PD [16].

Recently, cerebral dopamine neurotrophic factor (CDNF) was identified as a novel trophic factor for dopaminergic neurons. CDNF is expressed in several tissues including the mouse embryonic and postnatal brain. Interestingly, CDNF prevents 6-hydroxydopamine-induced degeneration of dopaminergic neurons in a rodent model of PD. Moreover, CDNF is almost as efficient as the glial cell line-derived neurotrophic factor (GDNF) in preventing the degeneration of dopaminergic neurons [14]. To investigate whether genetic variations of *CDNF* are related with

Abbreviations: CDNF, cerebral dopamine neurotrophic factor; PD, Parkinson disease; SNP, single nucleotide polymorphism; EOPD, early-onset Parkinson disease; GDNF, glial-cell line derived neurotrophic factors; OR, odd ratio; CI, confidence interval; MAF, minor allele frequency.

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PD, we performed mutation analysis of *CDNF* in early-onset PD (EOPD), and compared allele frequencies of selected *CDNF* single nucleotide polymorphisms (SNPs) between PD patients and controls.

We screened 110 unrelated EOPD patients (mean age at onset 40.53 ± 8.97 years, range 13–50 years, male:female = 52:58), for mutations in the *CDNF* gene. EOPD patients who were 50 years old or younger and signed an informed consent form were enrolled in this study. Fifteen EOPD patients had family history of PD. We included 215 PD patients, of which 110 were EOPD patients, for case–control association studies of *CDNF* SNPs. PD patients recruited in this study were diagnosed by 2 neurologists (YJK and HIM) according to the UK brain bank criteria [4]. PD patients were 13–89 years old (mean age, 55.28 ± 13.95 years); 40.94% were males. Age- and sex-matched normal controls without any neurological symptoms or signs on neurological examination were recruited from the National Health Examinee of the Hallym University Sacred Heart Hospital. Controls were 16–85 years old (mean 54.38 ± 14.33 years); 40.94% were males. All participants were Korean. The project was approved by the Institutional Review Board at the Hallym University.

To detect mutations in the *CDNF* gene, we directly sequenced all 4 exons. Peripheral blood was collected from each patient and genomic DNA was extracted from peripheral lymphoblasts according to a standard protocol. The exons of the *CDNF* gene were amplified with the GeneAmp PCR System 2700 (Applied Biosystems). Primer sequences and PCR conditions are listed in Supplementary Table 1. PCR products were electrophoresed on a 10% agarose gel at 100 V for 30 min and visualized by UV illumination after staining with ethidium bromide. Direct sequencing was performed by using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The electrochromatograms were analyzed with the Chromas software according to a reference sequence (NM ID: 001029954.2).

Analysis of the *CDNF* gene copy number was performed by using TaqMan® Copy Number Assays with a StepOne Plus RT-PCR system (Applied Biosystems). TaqMan® Copy Number Assays run a target DNA sequence (an exon of *CDNF*) simultaneously with a reference gene (*RNase P*, which exists in 2 copies in a diploid genome). Real-time fluorescent PCR was carried out in a reaction volume of 20 μL containing 25 ng genomic DNA and 250 nM probes. The StepOne Plus ABI system, TaqMan master mix, 96-well MicroAmp optical plates, and optical adhesive covers were all from Applied Biosystems. Each patient and control sample was amplified. PCR conditions were as follows: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min (40 cycles). The dosage of each ampli-primer relative to *RNase P* and normalized to control DNA was determined. Copy number variation was analyzed with the CopyCaller™ software. The CopyCaller™ software performs relative quantification analysis of threshold cycle (CT) data obtained by real-time PCR. The analysis determines the number of copies of the target sequence in each genomic DNA sample.

The SNPs analyzed in the case–control study between PD patients and controls were selected using the following method. The *CDNF* gene is located on chromosome 10p13. Nine SNPs (rs2278871, rs11818228, rs7900873, rs7099185, rs1901650, rs7094179, rs11814733, rs6602768, and rs11259365) located on exons, introns, 5′UTR, or 3′UTR of *CDNF* (from 14,901,257 bp to 14,919,989 bp of chromosome 10) were chosen. These SNPs are deposited in the International HapMap database (www.hapmap.org). PCR and direct sequencing analysis of these SNPs were performed in 26 controls using primers listed in Supplementary Table 2. We selected SNPs with minor allele frequency (MAF) greater than 5% and that did not deviate from the Hardy–Weinberg equilibrium (HWE).

Genotyping of SNPs in case–control association studies was performed by SNaPshot. Primers included in a single reaction need to differ significantly in length in order to avoid overlap between the final SNaPshot products. The length of SNP primers was 20-mer (rs7094179; gtt ttt tgt tcc agc tgg ct), 30-mer (rs11259365; gat cga tcg cca atg tat cat cta ggt gat), and 38-mer (rs1901650; gat cga tcg atc gat cgt gct cca gaa aac cct taa at). For multiplex PCR, 100 ng of DNA was amplified in a reaction mixture containing 2.5 mM dNTPs, 10× PCR buffer, *Taq* polymerase (iNtRON), and 100 ng of pooled forward and reverse primers. PCR amplification was performed in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the following conditions: 30 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s, followed by a final extension of 10 min at 72 °C. After PCR amplification, the PCR products were purified to remove excess dNTPs and primers using a modified protocol for ExoSAP (USB Corp.). To 5 μL of PCR product, 2 μL ExoSAP was added and incubated at 37 °C for 1 h, after which the enzyme was inactivated at 75 °C for 15 min. The purified products were stored at 4 °C. After postextension treatment, fresh tubes containing 9 μL highly deionized formamide (Applied Biosystems) and 0.5 μL GeneScan-120Liz size standard (Applied Biosystems) were prepared on ice. 0.5 μL SNaPshot multiplex PCR product were added to these mixtures. The DNA was denatured at 95 °C for 5 min and kept on ice before loading onto the ABI Prism 3100 genetic analyzer (Applied Biosystems, USA). Samples were run using POP-4 polymer, 50-cm capillaries, an injection time of 30 s, and an oven temperature of 60 °C. The results were analyzed using the GeneMapper software (version 3.5, Applied Biosystems) with the SNaPshot default analysis method. Comparison of genotype frequencies between cases and controls was assessed by χ^2 statistics. A two-tailed type-I error rate of 5% was chosen for the analysis.

Total RNA was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Germany) from peripheral lymphoblasts of control subjects according to the manufacturer's protocol. The integrity of the RNA was assessed using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). Only samples with RIN values higher than 0.9 were used for quantitative real-time PCR reaction. For cDNA synthesis, 0.1 μg total RNA was mixed with 1 μl Primer (Oligo(dT), 0.5 μg/reaction) using GoScript™ Reverse Transcription System (Promega, USA). Quantitative real-time PCR was performed by using Quanti Fast SYBR Green PCR Kit (Qiagen, Germany) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Real-time fluorescent PCR was carried out in a reaction volume of 20 μL containing 20 ng genomic DNA, 100 nM primers (CDNF-F: ctg tgc aga aaa aac tga cta tgt ga, CDNF-R: aaa tgt gct ggc att gga gat, GAPDH-F: ctg ctc ctc ctg ttc gac agt, GAPDH-R: ccg ttg act ccg acc ttc ac) and SYBR Green PCR master mix. For each primer pairs used for CDNF or GAPDH, the conditions were optimized so that melting or dissociation curve analyses showed a single melting peak after amplification. Comparison of the mRNA expression levels of CDNF among control groups with different genotypes of rs7094179 were performed by normalizing threshold cycle (C_T) values of CDNF to those of the housekeeping genes, GAPDH (C_T^{CDNF}/C_T^{GAPDH}).

No mutations or polymorphisms were identified in any of the exons analyzed in the 110 unrelated EOPD patients. We also analyzed gene dosage changes in all exons of *CDNF* in these patients by real-time PCR. However, no gene dosage changes suggestive of heterozygous deletion or duplication of exons in *CDNF* were found.

The genotype frequencies of 9 SNPs—deposited in the International HapMap database—were determined in 26 Korean controls. 4 SNPs (rs11815805, rs11818228, rs11814733, and rs6602768) were not polymorphic in the Korean population. Two SNPs (rs2278821 and rs7099185) deviated from HWE because of homozygote excess. For case–control association studies, we selected 3 SNPs (rs1901650, rs7094179, and rs11259365) that satisfied HWE and had MAF greater than 5%. The frequencies of all 3 analyzed SNPs in