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Screening for mutations of the HFE gene in Parkinson's disease patients with hyperechogenicity of the substantia nigra

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Abstract

Iron mediated oxidative stress is known to contribute to the neurodegenerative process in Parkinson's disease (PD). Although there are hints that genes involved in brain iron metabolism might be involved in the pathogenesis of PD in some instances, it is still not known whether the increase in brain iron content constitutes a primary or secondary event in the disease cascade. Recent studies on the role of *hemochromatosis gene* (*HFE*) mutations in PD vary from a protective effect of C282Y heterozygosity, no effect of the C282Y or H63D mutation to an increased risk for PD in C282Y mutation carriers. In this study, analyzing the whole coding region of the *HFE* gene by dHPLC in 278 PD patients, priorly characterized by transcranial sonography for increased iron content of the substantia nigra (SN), we did not find an association of the common *HFE* mutations and PD. However, we identified two novel variants (K92N and I217T) each in a single PD patient. These variations were not found in any of the controls. Future studies are necessary to reveal a possible functional relevance of these mutations for PD. Our results indicate that mutations in the *HFE* gene are not a common cause for PD with increased iron levels of the SN.

Keywords: Parkinson's disease; *Hemochromatosis gene* (*HFE*); Iron metabolism; Mutational screening; Transcranial sonography

A number of investigations have provided evidence for the contribution of iron-mediated oxidative stress to the pathogenesis of Parkinson's disease (PD) (reviewed in [1]). Although an increasing number of genes responsible for familiar monogenetic PD are being discovered, the vast majority of PD cases appears to be sporadic. It seems that these sporadic cases are either caused by single factors in individual families [15] or multifactorally with a genetic vulnerability contributing to disease manifestation. One group of genes of possible relevance are genes involved in brain iron metabolism [1].

An involvement of brain iron metabolism in the pathogenesis of PD is further supported by the demonstration of hyperechogenicity of the substantia nigra (SN) in PD patients, which is a marker for an increased iron level in about 90% of PD patients [4,22].

To detect a possible association of PD and/or increased SN-iron levels with variations in genes regulating brain iron metabolism we investigated 278 PD patients, priorly characterized by transcranial sonography (TCS) for SN echogenicity, for relevant mutations in the *hemochromatosis gene* (*HFE*).

Hereditary haemochromatosis (HH) is a genetically determined disorder in which disturbance of normal iron metabolism leads to systemic iron accumulation. Although HH is usually not associated with neurological symptoms and iron deposition in the brain seems to occur only rarely, there are several reports on patients with HH, basal ganglia iron deposition and the clinical presentation of idiopathic PD [11,18], rendering the possibility of induction of PD by abnormal deposition in the basal ganglia likely. The highly penetrant C282Y mutation leading to intracellular iron sequestration and the less penetrant H63D mutation constituting rather a risk factor, account for most cases of HH. Recent studies on the role of *hemochromatosis gene* mutations in PD vary from a protective effect of C282Y heterozygosity [7], no effect of either mutation [6] to an increased risk for PD

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in C282Y mutation carriers [12]. None of these studies was designed to look for other possible mutations in the *HFE*. We therefore set out to screen the whole encoding region of the *HFE* in PD patients with evidence for increased iron levels of the SN.

Two hundred seventy eight PD patients (median age 64 (60; 72) years, 161 males, 117 females) with idiopathic PD according to the UK brain bank criteria agreed to participate in this study and gave informed consent according to the declaration of Helsinki. Additionally, 280 ethnically matched controls (median age 57 (52; 60) years; 143 males, 137 females) without extrapyramidal disorders were included.

All patients and controls underwent a thorough neurological investigation and transcranial ultrasound examination (TCS). Blood samples were taken for genetic analyses.

TCS examination was performed in a standardized examination technique with a color-coded, phased-array ultrasound system equipped with a 2.5-MHz transducer (Elegra, Siemens, Erlangen, Germany) as described previously [3]. As signal brightness (echogenicity) of the SN generated by ultrasound is not quantifiable, the area of hyperechogenic signals in the SN-region was encircled and measured. An area of SN echogenicity exceeding 0.19 cm^2 was classified as hyperechogenic, a value representing the upper standard deviation of healthy subjects [4].

PCR amplification for every exon of the *HFE* gene was carried out in a thermocycler (GeneAmp PCR System 9700; Applied Biosystems) under the following conditions: 250 ng DNA was amplified in a final volume of 25 μl in the presence of 16 mM (NH4) 2 SO 4, 67 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 100 μM of each dNTP, 400 nM of each PCR primer and 2 U Taq Polymerase (BioTherm™ DNA Polymerase, Genecraft). Standard cycling conditions were performed with optimized annealing temperatures (T_A; Table 1): initial denaturating step of 5 min at 95 °C; 30 cycles (94 °C for 30 s; T_A for 60 s and 72 °C for 60 s) followed by a final elongation step at 72 °C for 10 min. PCR primer pairs were constructed with the online software Pimer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) with the aid of the published genomic sequence of the *HFE* gene (Table 1). Primer sequences and respective optimized annealing temperatures are given in Table 1.

Table 1
Oligonucleotide primers, PCR product size, PCR annealing temperatures (T_A) and dHPLC column temperatures (T_C) used for the analysis of the *HFE* gene (Acc. number NT_007592)

HFE	Primer-sequence 5′–3′	Size (bp)	Annealing T_A (°C)	dHPLC T_C (°C)
Exon 1 forward	ggt gac ttc tgg agc cat cc	339	55	65.3
Exon 1 reverse	ccc aag cgc aaa gaa aaa g			
Exon 2 forward	cta cac atg gtt aag gcc tg	389	55	59.7
Exon 2 reverse	acc tca gac ttc cag ctg tt			
Exon 3 forward	ggt tgc agt taa caa ggc tg	360	55	59
Exon 3 reverse	tcc tcc act ctg cca cta ga			61.5
Exon 4 forward	aga tcc cct ctc ctc atc ct	484	55	59.3
Exon 4 reverse	ttt tct cag ctc ctg gct ct			61
Exon 5 forward	agt gag atg agg atc tgc tc	457	55	57
Exon 5 reverse	cac agt gac ttc tca acc tc			59
Exon 6 forward	aga gaa gag gca aga tgg tg	235	55	61.5
Exon 6 reverse	caa gga gtt cgt cag gca at			

Exon 3, 4 and 5 were investigated at two different dHPLC column temperatures.

DHPLC mutation screening was performed for every PCR product on a Wave DNA Fragment Analysis system (Transgenomic) with temperatures as described in Table 1. Conditions were ascertained using the Wavemaker Software (Version 4.1.31, Transgenomic).

Prior to the analysis, two PCR products were pooled at equimolar ratio, denatured at 94 °C for 2 min and reannealed over 30 min by gradual cooling from 94 °C to 10 °C to allow the formation of hetero- and homoduplexes. In the case of a suspicious dHPLC elution profile, each of the pooled samples was tested separately for detection of heteroduplices. Some exons were screened at two different temperatures as shown in Table 1. DNA samples exhibiting heteroduplexes were sequenced on a CEQ 8000 cycle sequencer using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckmann Coulter, Krefeld, Germany) with the same primers used for the PCR (Table 1).

For descriptive statistics, median with lower and upper quartile are given. Patients' characteristics were compared by the nonparametric Mann–Whitney-*U* test. Data for the different loci were evaluated for allele frequencies, genotype frequencies and Hardy–Weinberg equilibrium using the Genepop version 3.3. at http://wbiomed.curtin.edu.au/genepop. Significance testing of genotype frequencies for the two groups was conducted using Fisher's exact test. Moreover, odds ratios adjusted for age using logistic regession modelling were calculated.

TCS examination revealed a median area of SN echogenicity of 0.25 (0.21; 0.28) cm^2 for the left and of 0.23 (0.20; 0.26) cm^2 for the right side in PD patients versus 0.12 (0.08; 0.16) cm^2 for the left and 0.12 (0.08; 0.16) cm^2 for the right side in controls. Differences between the groups were significant for both sides (*U* test, $p < 0.001$).

Using dHPLC, we identified a number of suspicious elution profiles of the *HFE* in PD patients. Sequencing of the respective samples revealed two novel variations, K92N (c.3821 A > C) and I217T (c.5499 C > T) in exons 2 and 4, respectively. The PD patients harbouring these mutations both had the clinical picture of typical dopa-responsive idiopathic PD and hyperechogenicity of the SN. There was no phenotypic conspicuousness concerning severity or disease progression compared to the other patients with sporadic PD. Both variants were not demonstrable in 524 control alleles. For the H63D and C282Y polymorphisms, commonly causing HH, no significant differences of genotype frequencies between patients and controls (Table 2; Fisher's exact test, $p > 0.05$) were found. This result was confirmed after exclusion of patients without increased echogenicity of the SN and controls with increased echogenicity of the SN from the analysis.

Calculation of odds ratios were non-significant for both variants. Odds ratio of the homozygeous possession of the 282Tyr allele was 0.19 (95% CI = 0.02–1.64, $p = 0.13$) and 0.25 (95% CI = 0.03–2.23, $p = 0.2$) after logistic regression analyses, correcting for age. For heterozygeous possession the respective values were1.09 (95% CI = 0.59–2.04, $p = 0.78$) and 1.31 (95% CI = 0.67–2.57, $p = 0.43$). Odds ratio of the homozygeous possession of the 63Asp allel was 0.99 (95% CI = 0.31–3.12,