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PARK2 variability in Polish Parkinson's disease patients - interaction with mitochondrial haplogroups

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Abstract

Aims and objectives—A new pathomechanism of Parkinson's disease (PD) involving regulation of mitochondrial functions was recently proposed. Parkin complexed with mitochondrial transcription factor A (TFAM) binds mtDNA and promotes mitochondrial biogenesis, which is abolished by *PARK2* gene mutations. We have previously shown that mitochondrial haplogroups/clusters and *TFAM* common variation influenced PD risk. We investigate the role of *PARK2* polymorphisms on PD risk and their interactions with mitochondrial haplogroups/clusters as well as with *TFAM* variability.

Methods—104 early-onset PD patients (EOPD, age at onset < 50 years) were screened for *PARK2* coding sequence changes including gene dosage alterations. Three selected *PARK2* polymorphisms (S167N, V380L, D394N) were genotyped in 326 PD patients and 315 controls using TaqMan allelic discrimination assay.

Results—*PARK2* screen revealed two heterozygous changes in two EOPD patients: exon 2 deletion and one novel synonymous variation (c.999C>A, P333P).

In association study no differences in genotype/allele frequencies of S167N, V380L, D394N were found between analyzed groups. Stratification by mitochondrial clusters revealed higher frequency of V380L G/G genotype and allele G in PD patients, within HV cluster ($p=0.040$; $p=0.022$, respectively). Moreover, interaction between genotypes G/G V380L of *PARK2* and G/G rs2306604 of *TFAM*, within HV cluster was significant (OR 2.05; CI 1.04 – 4.04; $p=0.038$).

Conclusions—Our results indicate that co-occurrence of G/G V380L *PARK2* and G/G rs2306604 *TFAM* on the prooxidative HV cluster background can contribute to PD risk. We confirm low *PARK2* mutation frequency in Polish EOPD patients.

Keywords

Parkinson's disease risk factors; *PARK2*; mitochondrial clusters; mitochondrial transcription factor A (TFAM)

Introduction

PARK2 is a large gene, spanning 1.3 Mb, with almost 200 known mutations scattered over all twelve exons [1]. They comprise point mutations, small insertions/deletions and exon rearrangements [2]. Homozygous and compound heterozygous *PARK2* mutations are the most frequent cause of autosomal recessive early onset Parkinson disease (EOPD). They are found in about 1% of LOPD (late onset PD) patients (age at onset - AAO>50 years) [3]. Single heterozygous *PARK2* mutations were also implicated in PD pathogenesis [3,4]. Moreover, numerous association studies addressed the role of common *PARK2* polymorphisms in PD risk with often contradictory results [esupp1–9]. There is an ongoing debate whether *PARK2* mutations associated PD overlaps pathophysiologically with sporadic PD [esupp10–11].

Various molecular mechanisms were proposed to underlie pathogenic effects of *PARK2* mutations [5]. The primary hypothesis focused on ubiquitin-proteasome dysfunction due to impairment of ubiquitin E3 ligase activity of parkin [6]. There is growing evidence that parkin directly regulates mitochondrial function [7–9]. Independent studies showed that parkin complexed with TFAM, binds to mtDNA, increasing mitochondrial transcription and replication, which is abolished by pathogenic *PARK2* mutations [10,11]. Physical interaction between parkin and mtDNA was shown both *in vivo* and *in vitro* [10,11]. These results strongly suggest that *PARK2* variants compromising parkin function could result in mitochondrial dysfunction, leading to PD pathology.

A few association studies corroborated the protective role of specific mitochondrial DNA haplogroups/clusters against PD [12–15]. *In silico* and *in vivo* analyses suggested that mtDNA sublineages U4, U5a1, K, J1c and J2 within JTKU cluster, are characterized by haplogroup-specific polymorphisms decreasing mitochondrial coupling efficiency, which lead to reduced reactive oxygen species generation [16,17]. Indeed, we reported previously that haplogroup J and U4U5a1KJ1cJ2 group were associated with decreased PD risk (OR 0.581, CI 0.340–0.995, $p = 0.0476$; OR 0.647, CI 0.440–0.950, $p = 0.0259$, respectively) in our subjects [15]. On the other hand, we found that haplogroup H and HV cluster increased the risk of Alzheimer's disease (AD), but not PD in our cohort, probably via increased ROS production [15,18]. We have also reported that an intronic *TFAM* polymorphism (rs2306604, IVS4 + 113A > G) is an independent PD risk modifier, possibly interacting with mtDNA HV cluster [19].

Taking into consideration the crucial role of parkin in mitochondrial maintenance and PD pathogenesis, we hypothesized that potentially functional *PARK2* variants might influence PD risk depending on mitochondrial haplogroup/cluster background.

First, we screened 104 EOPD patients for *PARK2* changes, to identify putative disease causing mutations and polymorphisms in Polish population. Then we chose *PARK2* polymorphisms observed in this screen with a frequency of the minor allele >2% (S167N, V380L, D394N) to test the above stated hypothesis in 326 PD patients and 315 control subjects and in subgroups stratified by mitochondrial haplogroup clusters (HV, JTKU) and a functional group U4U5a1KJ1cJ2, characterized by the partial uncoupling of oxidative phosphorylation.

Methods

Subjects

The studied group consisted of 326 PD patients (mean age at onset (AAO), 55.6 ± 12.8 years; mean age at enrollment (AAE) 62.4 ± 12.4 years, range: 23–80 years, 52% females) and 315

control subjects (AAE: 70.5 ± 6.8 years, range: 47–90 years, 61% females). All patients met UK PD Society Brain Bank clinical diagnostic criteria. Cases with secondary and atypical parkinsonism and patients taking neuroleptics and/or dopaminolytic drugs were excluded. AAO was self-reported by the PD patients. EOPD patients were selected on the basis of age at onset (AAO ≥ 50 years). Control group consisted of subjects without history of neurological disorders and without neurological abnormalities at examination (dementia was excluded by Mini Mental State Examination, only controls with MMSE > 28 were included). All cases and controls were Caucasians of Polish origins, recruited in 5 Polish movement disorders centers. The study was approved by the Ethics Committees of each participating institution and written informed consent was obtained from all participants.

Molecular analysis

DNA was isolated from peripheral blood lymphocytes using standard procedures.

All exons and intron-exon boundaries of the *PARK2* gene were screened in a subgroup of 104 patients presented with EOPD (AAO ≥ 50 years: 43.5 ± 5.8 years; 43% females) by direct sequencing using standard methods on ABI3700 (Applied Biosystems, Foster City, CA, USA) and analysis was performed with SeqScape v2.5 software.

Detection of gene dosage alterations in the *PARK2* gene was performed using SALSA MLPA Kit P051-C1/P052-C1 Parkinson (MRC-Holland, Amsterdam, Netherlands). Due to insufficient DNA quantities MLPA was performed in 80 EOPD cases (AAO ≥ 50 years: 43.2 ± 6.1 years; 43.8% females). Data analysis was done with GeneMapper software v.4.0 (Applied Biosystems).

Three *PARK2* variants with a frequency of the minor allele $> 2\%$ (S167N, rs1801474; V380L, rs1801582 and D394N, rs1801334) were genotyped using TaqMan assay (ABI 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA) in 326 PD patients and 315 controls.

Mitochondrial haplogroups and subhaplogroups were analyzed as described previously [15].

Analysis *in silico*

The effect of the identified silent exonic DNA variation on splicing was investigated with ESEfinder (vs. 3.0), enabling identification of putative exon splicing enhancers responsive to the human serine/arginine-rich (SR) proteins [esupp12–13].

Statistics

Chi-square test or two-tailed Fisher's exact test was used to assess differences in frequency distributions of *PARK2* genotypes, alleles and haplotypes between PD patients and controls. Univariate and bivariate logistic regression with interaction analysis was additionally applied to assess association between PD, *TFAM* genotypes and mitochondrial clusters/groups. Reported p values were not adjusted for multiple testing. Statistical significance was established at $p < 0.05$. Statistical analyses were performed using STATISTICA 6.0.

The statistical power for comparison of our PD (N= 326) and control (N= 315) groups was sufficient to detect with 80% probability true differences of the *PARK2* genotype frequencies from 2% to 11% for the most rare and the most common genotypes, respectively. The differences for 90% statistical power were from 2.5% to 13%, respectively.

Results

Sequence analysis of all 12 *PARK2* exons in 104 EOPD patients revealed one novel, heterozygous silent substitution in exon 9, c.999C>A, (P333P), in one female patients (AAO=49 years, frequency 1%). *In silico* analysis indicated that the nucleotide substitution potentially modifies the pattern of binding sites for specific serine/arginine-rich (SR) proteins, that take part in proper exon recognition during splicing. P333P decreases the anticipated strength of SC35 binding, increases the strength of SF2/ASF (IgM-BRCA1) (1) binding, disrupts another SF2/ASF (IgM-BRCA1) (2) binding site; an additional site for SF2/ASF protein in the same position as SF2/ASF (IgM-BRCA1) (1) appears, however, simultaneous binding of two overlapping ESEs is considered as little probable [esupp14] (Table 1, supplementary).

Gene dosage analysis revealed one, single heterozygous exon 2 deletion in one female patient with AAO = 50 years (1.7%).

Additionally six known, polymorphisms S167N, L307L, V380L, D394N, R402C and C451Y were identified (Table 2, supplementary).

For association analysis in 326 PD patients and 315 control subjects we chose the *PARK2* polymorphisms with a frequency of the minor allele >2% (S167N, V380L, D394N).

The distributions of *PARK2* genotypes of analyzed polymorphisms are in Hardy-Weinberg equilibrium (HWE, not shown). No differences were found in *PARK2* genotype distribution between mitochondrial haplogroups, neither among PD patients, nor in controls; between EOPD cases and LOPD (AAO>50 years) cases, and also after stratification into HV and JTKU cluster, so these patients were included into one group for further analyses. We have found no differences in genotype frequencies of S167N, V380L, D394N between PD patients and controls (Table 1). However, after stratification by haplogroup clusters we found higher frequency of genotype G/G V380L (rs1801582) in PD patients than in controls within HV cluster, but not in JTKU cluster and U4U5a1KJ1cJ2 subgroup (Table 1; $p=0.054$). Further univariate logistic regression analysis indicated that genotype G/G V380L significantly increased PD risk within HV cluster (Table 2). We found a borderline interaction between V380L G/G genotype effect and HV cluster effect ($p = 0.065$) in bivariate logistic regression model (Table 2).

There was no interaction between V380L G/G genotype and gender in bivariate logistic regression model ($p = 0.99$).

No differences in allele frequencies for S167N and D394N between PD and control groups were found (respectively: allele T 3.2% in PD patients, 2.5% in controls, $p=0.51$; allele T 5.7% PD patients, 4.3% in controls, $p=0.30$, Fisher's exact test). However, allele G of V380L, occurred with higher frequency in PD patients within HV cluster ($p=0.022$, Table 3, supplementary material). Further univariate logistic regression analysis indicated that allele C, of V380L decreased PD risk only within HV cluster (OR 0.605, 95% CI 0.374–0.980, $p=0.040$).

We have previously shown that rs2306604 G/G genotype of *TFAM* is an independent risk factor for PD (OR 1.789, 95% CI 1.162–2.755, $p=0.008$). There was a borderline interaction between G/G genotype and cluster HV ($p = 0.065$) [19].

In order to further characterize the interplay between nuclear and mitochondrial PD risk factors, we simultaneously analyzed V380L *PARK2* and rs2306604 *TFAM* genotypes within HV cluster. We found that combined genotype: rs2306604 G/G of *TFAM* and V380L

G/G of *PARK2*, was associated with increased PD risk, within mitochondrial HV cluster, in comparison to the most common *TFAM*rs2306604 (A/G or A/A) and *PARK2* V380L G/G combination (OR 3.94, 95%CI 1.82–8.53, $p=0.0003$, Table 3), as well as in comparison to all other genotype combinations pooled (OR 4.33, 95%CI 2.055–9.118, $p=0.00004$). There were no significant differences in frequencies of these 3 genotype combinations (other than G/G-G/G) between PD and control groups ($p=0.67$, χ^2 test). The interaction between rs2306604 G/G genotype of *TFAM* and V380L G/G genotype of *PARK2*, within mitochondrial HV cluster was significant in bivariate logistic regression model ($p=0.038$, Table 4).

Discussion

According to primary estimations *PARK2* mutations were responsible for about 18% of sporadic EOPD cases, reaching up to 50% of early onset familial cases with recessive inheritance in European populations [supp15–16]. Further analyses verified this data indicating that *PARK2* mutation frequency is highly population-specific. The apparent discrepancies between studies arose due to clinical heterogeneity of analyzed cohorts, characterized by different proportion of familial vs. sporadic EOPD cases, and different cutoff point for the AAO. Indeed, there are populations with very low *PARK2* mutation frequency [20–22]. Our results confirm low frequency of *PARK2* mutations in Polish EOPD patients [21]. We found two single heterozygous *PARK2* changes, one already described exon 2 deletion and one novel, silent variation P333P (c.999C>A), in exon 9, that is alternatively spliced in TV4, TV5 and TV7 isoforms [23]. *In silico* analysis predicted that the point mutation modifies the pattern of putative exonic splicing enhancers responsive to specific SR proteins, that take part in proper exon recognition during splicing. Single heterozygous *PARK2* mutations are found with similar frequency in PD patients and in healthy individuals and their role in PD pathogenesis is not well understood [3]. Positron emission tomography (PET) imaging showed nigrostriatum dysfunction in asymptomatic single heterozygous *PARK2* mutation carriers [4]. Three mechanisms are postulated to underlie PD caused by single *PARK2* mutations: haploinsufficiency, dominant negative effect, and gain of function [3]. Additive effect of two single heterozygous mutations in different genes should also be considered [20].

There are conflicting results of association studies concerning the role of *PARK2* polymorphisms [1, supp1–9, supp17] (also Table 4, supplementary material). Our results point to the V380L as a factor influencing PD risk. Amino acid position 380 is located in the IBR domain of parkin. Structural analyses indicate that the domain is likely responsible for stabilizing the geometry and orientation of the two RING domains within parkin [24]. Thus, V380L change may result in decreased interaction with E2 protein–ubiquitin-conjugating enzymes, but also in impaired binding and ubiquitination of substrates, like synphilin-1 and p38 [24].

Our association study, for the first time, analyzed the interaction between *PARK2* polymorphisms and 1). mitochondrial clusters and 2). *TFAM* variation. Our results suggest that G/G genotype of V380L is PD risk factor in combination with the prooxidative background of the most common mitochondrial HV cluster. We have previously noticed a similar tendency, for genotype G/G rs2306604 *TFAM* and HV cluster [19].

Our study, similarly to the meta-analysis of V380L on PDmutDB webpage (allele C vs. G) indicates no effect on PD risk in Caucasian population (OR 0.92, 95% CI 0.78–1.08) [1]. However, the overall meta-analysis, comprising all ethnicities and studies where HWE was violated in control groups, suggests that allele C is protective against PD [1]. In our data, allele C of V380L decreases PD risk only on HV cluster background. Previous reports

pointed to G/G V380L genotype as a factor increasing PD risk or allele C as a protective one [5,6]. Among PD patients potentially exposed to pesticides, postulated to increase PD risk, those carrying allele C of V380L had higher age at onset [4] (Table 4, supplementary material). Interestingly, this allele also decreases the risk of familial and sporadic progressive supranuclear palsy (PSP), a tauopathy, which on genetic, clinical and histopathological level partially overlaps with PD [25].

We also found that the interaction between rs2306604 G/G genotype of *TFAM* and V380L G/G genotype of *PARK2*, within mitochondrial HV cluster was significant in bivariate logistic regression model ($p=0.038$). This suggests that simultaneous presence of G/G V380L *PARK2* and G/G rs2306604 *TFAM* on HV cluster background is a PD risk factor, with detrimental influence more powerful than predicted by additive model.

It was recently proposed that haplogroup H and HV cluster increase the risk of neurodegenerative diseases due to higher coupling ability resulting in increased ROS production [18,26]. On the other hand, functional studies revealed that parkin directly regulates vital mitochondrial functions, promoting mtDNA transcription, replication, as well as mtDNA repair [10,11]. Although animal PD models based on *PARK2* deficiency recapitulated hardly any PD-like features, they indeed demonstrated increased oxidative stress along with some impaired mitochondrial parameters [7]. Parkin was also proposed to promote autophagy of damaged mitochondria [27]. Thus, we can envision that functional *PARK2* polymorphic changes, even slightly decreasing mitochondrial function, could ultimately contribute to neurodegeneration.

It could be also speculated that *PARK2* polymorphisms, or polymorphisms located in other genes related to mitochondrial function, could modulate PD risk, depending on mitochondrial genetic background [19,28].

However, our study has several limitations. First, we evaluated multiple hypotheses and only one observed association (increased PD risk by simultaneous occurrence of genotype GG *PARK2* V380L and GG *TFAM* rs2306604 within mitochondrial HV cluster, see Table 3) would remain statistically significant after stringent Bonferroni correction for multiple comparisons (since one *TFAM*, three *PARK2* SNPs and three possible *TFAM-PARK2* SNPs interactions were analyzed in five mitochondrial groups, the corrected p-value threshold is $0.05/[5 \times (1+3+3)] = 0.05/35 = 0.0014$). Moreover, the interaction between G/G V380L genotype and mtDNA HV cluster shows only a trend ($p=0.065$). Our sample size was sufficient for a single effect analysis, but many times bigger population sample would be necessary (a few thousands subjects) to perform interaction analyses with equivalent power, and to confirm unambiguously the interactions between *PARK2* versus *TFAM* and mtDNA haplogroups. On the other hand, functional studies could contribute to better understanding of *PARK2*, *TFAM* and mtDNA variation interplay and their involvement in PD pathogenesis. Recent findings in cybrid models demonstrated that mitochondrial haplogroups influenced mtDNA replication and transcription, and mitochondrial and nuclear genes expression pattern, involved in oxidative phosphorylation pathway [29,30]. Mitochondrial parameters were also changed in fibroblasts and leukocytes derived from EOPD patients carrying *PARK2* mutations [8,9]. Analogous approach, based on detailed characterization of mitochondrial function as well as oxidative stress markers and antioxidant enzymes levels in PD patients and healthy controls with well defined genetic background (mtDNA haplogroups, *PARK2* and *TFAM* variants, we suggested as PD risk factors) could potentially confirm the hypotheses by us proposed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Genotype frequencies of three *PARK2* polymorphisms in PD patients and controls.

Polymorphism/genotype	Total PD n=326	Total control n=315	PD HV cluster n=149	Controls HV cluster n=151	PD non-HV group n=177	Controls non-HV group n=164	PD JTKU cluster n=134	Controls JTKU cluster n=142	PD U4U5a1KJ1c1J2 n=57	Controls U4U5a1KJ1c1J2 n=78
n (%)										
S167N (rs1801474), Exon4										
C/C	305 (93.6)	300 (95.2)	141 (94.6)	143 (94.7)	164 (92.7)	157 (95.7)	123 (91.8)	135 (95.1)	54 (94.7)	74 (94.9)
C/T	21 (6.4)	14 (4.4)	8 (5.4)	7 (4.6)	13 (7.3)	7 (4.3)	11 (8.2)	7 (4.9)	3 (5.3)	4 (5.1)
T/T	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
p	0.32		0.59		0.23		0.27		1.0	
V380L (rs1801582), Exon 10										
G/G	228 (69.9)	207 (65.7)	106 (71.1)	90 (59.6)	122 (68.9)	117 (71.3)	92 (68.7)	101 (71.1)	42 (73.7)	59 (75.6)
G/C	89 (27.3)	96 (30.5)	41 (27.5)	54 (35.8)	48 (27.1)	42 (25.6)	38 (28.4)	36 (25.3)	15 (26.3)	16 (20.5)
C/C	9 (2.8)	12 (3.8)	2 (1.3)	7 (4.6)	7 (3.9)	5 (3.0)	4 (3.0)	5 (3.5)	0 (0.0)	3 (3.8)
p	0.47		0.054		0.84		0.84		0.26	
D394N (rs1801334), Exon 11										
C/C	292 (89.6)	288 (91.4)	135 (90.6)	141 (93.4)	157 (88.7)	147 (89.6)	120 (89.5)	130 (91.5)	53 (92.9)	72 (92.3)
C/T	31 (9.5)	27 (8.6)	13 (8.7)	10 (6.6)	18 (10.2)	17 (10.4)	12 (9.0)	12 (8.4)	3 (5.3)	6 (7.7)
T/T	3 (0.92)	0 (0.0)	1 (0.7)	0 (0.0)	2 (1.1)	0 (0.0)	2 (1.5)	0 (0.0)	1 (1.7)	0 (0.0)
p	0.14		0.47		0.39		0.34		0.44	

p –Chi2 test for global association of a given polymorphism with PD risk.

Table 2

Odds ratios (ORs) and 95% confidence intervals (95% CIs) for V380L genotypes as PD risk factors in different haplogroup clusters.

Haplogroups/clusters	Genotypes	PD patients vs. control group		
		OR	95% CI	p [*]
Univariate logistic regression of V380L				
All subjects	GG vs. GC + CC	1.21	0.87–1.70	0.27
	GG +GC vs. CC	1.39	0.58–3.34	0.51
	GG vs. CC	1.47	0.60–3.57	0.50
HV cluster	GG vs. GC + CC	1.67	1.03–2.71	0.040
	GG +GC vs. CC	3.57	0.72–17.63	0.17
	GG vs. CC	4.12	0.83–20.54	0.088
JTKU cluster	GG vs. GC + CC	0.89	0.53–1.48	0.69
	GG +GC vs. CC	1.19	0.31–4.54	1.00
	GG vs. CC	1.14	0.29–4.42	1.00
U4U5a1KJ1cJ2 ^{***}	GG vs. GC + CC	0.92	0.41–2.07	0.84
	GG +GC vs. CC	∞	-	0.26
	GG vs. CC	∞	-	0.27
Bivariate logistic regression of G/G V380L genotype and HV cluster				
All subjects	HV vs. non-HV	0.82	0.59–1.15	0.26
	GG vs. GC + CC	1.22	0.87–1.70	0.25
	HV×GG ^{**}	1.37	0.98–1.92	0.065

* - Fisher's exact test for univariate analysis and Wald statistics for bivariate logistic regression analysis.

** - HV×GG designates interaction between HV cluster and GG V380L genotype.

*** - putative uncoupling subhaplogroups

Table 3

Frequencies, odds ratios (ORs) and 95% confidence intervals (95% CIs) for genotype combinations of *PARK2* V380L and *TFAM* rs2306604 within mitochondrial HV cluster in PD patients and controls.

<i>PARK2</i> V380L	<i>TFAM</i> rs2306604	PD HV cluster n=149	Controls HV cluster n=151	OR*	95%CI	p
		n (%)	n (%)			
GG	AG or AA	71 (47.6)	80 (53.0)	1	reference combination	
GC or CC	AG or AA	37 (24.8)	52 (34.4)	0.80	0.47–1.36	0.42
GC or CC	GG	6 (4.0)	9 (6.0)	0.75	0.25–2.21	0.79
GG	GG	35 (23.5)	10 (6.6)	3.94	1.82–8.53	0.00030

* OR, 95%CI and p values for comparison with the most common *PARK2* V380L GG - *TFAM* rs2306604 (AG or AA) genotype combination treated as a reference one

Table 4

Bivariate logistic regression of G/G V380L of *PARK2* genotype and G/G rs2306604 of *TFAM* within mitochondrial HV cluster.

Risk factor/interaction	OR	95% CI	p
<i>PARK2</i> V380L G/G	2.56	1.30–5.04	0.0066
<i>TFAM</i> rs2306604 G/G	1.92	0.98–3.79	0.059
<i>PARK2</i> V380L G/G $\times$ <i>TFAM</i> rs2306604 G/G *	2.05	1.04–4.04	0.038

* – interaction between genotypes *PARK2* V380L G/G, and *TFAM* rs2306604 G/G.