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Analysis of *Leucine-rich repeat kinase 2* (*LRRK2*) and *Parkinson protein 2* (*parkin, PARK2*) genes mutations in Slovak Parkinson disease patients

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Abstract. Parkinson disease (PD) is a chronic neurodegenerative movement disorder characterized by selective loss of nigrostriatal dopaminergic neurons and formation of Lewy bodies. Clinical manifestations include motor impairments involving tremor, bradykinesia, postural instability and rigidity.

Using dHPLC method we screened exons 31, 35, 41, 48 of the *Leucine-rich repeat kinase 2* (*LRRK2*) gene and exons 2, 6 and 7 of *Parkinson protein 2* (*parkin*, *PARK2*) genes in a cohort of 216 consecutive, unrelated Slovak patients with familial or sporadic PD, including early and late onset. By this means we aimed to detect the most common pathogenic mutations within *LRRK2 (*Arg1441Cys, Arg1441Gly, Arg1628Pro, Tyr1699Cys, Gly2019Ser, Ile2020Thr, Gly2385Arg*)* and *parkin* genes responsible for late and early onset forms of disease, respectively. However, none of these mutations was identified in our cohort. Heterozygous point mutation p.Arg275Trp in exon 7 of *parkin* gene was identified in one patient with age at onset 61 years. Furthermore, we observed the presence of one exonic (*LRRK2* ex 48: 7155A>G) and eight intronic polymorphisms (in *LRRK2*: IVS35+23T>A, IVS47-91insGCCAT, IVS47-91insGCAT, IVS47-41A>G, IVS47-9delT, IVS47-20C>T, IVS47-90A>G, in *parkin*: IVS2+25T>C), three of which were novel.

Key words: dHPLC — p.Arg275Trp — Parkinson disease — *Parkin* polymorphism — *LRRK2* polymorphisms

Introduction

The Parkinson's disease (PD) is the second most common progressive neurodegenerative brain disorder caused by loss of nigrostriatal dopaminergic neurons, which affect the control of body movements, with formation of inclusions (Lewy bodies) in surviving neurons. It affects 1–2% of the global population above 65 years and its prevalence increases to approximately 4% in those above 85 years (Calne et al. 1992; Polymeropoulos et al. 1996; Nussbaum et al. 1997; Biswas et al. 2006). The disease is characterized by bradykinesia, resting tremor, rigidity and postural instability (Nussbaum et al. 1997) and it is generally considered to be the result of the interaction between genetic and environmental factors (Calne et al. 1987; Eriksen et al. 2005).

Mutations responsible for the recessive early-onset PD (EOPD) were identified in *parkin* (*PRKN*, *PARK2*; MIM 602544) (Kitada et al. 1998), *DJ-1* (*PARK7*; MIM 602533) (Bonifati et al. 2003) and *PTEN-induced kinase 1* (*PINK1*, *PARK6*; MIM 608309) (Valente et al. 2004), while mutations within *Leucine-rich repeat kinase 2* (*LRRK2*, *PARK8*; MIM 609007) (Paisan-Ruiz et al. 2004; Zimprich et al. 2004a) and *α-synuclein* (*SNCA*, *PARK1*, *PARK4*; MIM163890) (Polymeropoulos et al. 1997) are associated with autosomal dominant, late onset form of PD.

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Leucine-rich repeat kinase 2 (LRRK2, PARK8) gene

The *PARK8* locus containing approximately 116 genes was mapped to chromosome position 12q in a large Japanese

family presenting with autosomal dominant parkinsonism (Funayama et al. 2002) and later on this finding was confirmed also in other countries. The *PARK8*-linked families are characterized by late onset of the disease, i.e. over 50 years (Funayama et al. 2002; Paisan-Ruiz et al. 2004; Zimprich et al. 2004b; Bialecka et al. 2005; Di Fonzo et al. 2006a).

The *LRRK2* gene covers 145 kb of genomic DNA and is split into 51 exons that encode for a large 2527 amino acid protein dardarin (derived from "dardara", the Basque word for tremor) that belongs to a group of the Ras/GTPase superfamily, termed ROCO. This protein is highly conserved among vertebrates and is multifunctional with five functional domains: LRR (Leucine-rich repeat), Ras GTPase (Roc), COR (C-terminal of Roc), MAPKKK (tyrosine kinase-like mitogen-activated protein kinase kinase kinase) and a WD-40 (Zimprich et al. 2004a). Dardarin is functionally implicated in substrate binding, phosphorylation and protein–protein interactions (Moore et al. 2005; Punia et al. 2006).

LRRK2 is postulated to be a key molecule in the etiology of both familial and sporadic PD. However, the molecular mechanism whereby mutant LRRK2 causes loss of dopaminergic neurons has not yet been confirmed. This is due to the lack of consensus about the true physiological substrate or true function of LRRK2. Ohta et al. (2011) demonstrated for the first time that LRRK2 directly phosphorylates serine/threonine kinase Akt1 (also known as protein kinase B1) that is the central molecule that transduces signals from receptors for growth factors and hormones to insure cell survival and protection of cells from apoptosis. Ser473, one of two amino acids essential for Akt1 activation, was the target site for LRRK2 (Dudek et al. 1997; Manning and Cantley 2007).

The large number of missense mutations has been found within *LRRK2* with varying degrees of proof of pathogenicity. The disease-associated mutations R1441C, G2019S and I2020T exhibited reduced interaction with and phosphorylation of Akt1, suggesting one possible mechanism for the neurodegeneration caused by *LRRK2* mutations (Ohta et al. 2011).

Some mutations are surprisingly common: R1441G is found in a large proportion of Basque cases (Simón-Sánchez et al. 2006), G2019S in Europeans, especially among those with Semitic ancestry (Kachergus et al. 2005; Ozelius et al. 2006) and both G2385R (Mata et al. 2005; Di Fonzo et al. 2006a; Tan et al. 2007; Choi et al. 2008) and R1628P (Ross et al. 2008) explain a large proportion of disease among eastern Asian peoples. The penetrance of all the mutations is not clear, but the G2019S mutation is nearly fully penetrant by the age of 80 (Kachergus et al. 2005; Healy et al. 2008).

Parkinson protein 2 (PARK2, parkin) gene

An autosomal recessive form of familial juvenile parkinsonism (AR-JP, *PARK2*; OMIM 602544), defined as onset before age 40, was described in a Japanese family by Takahashi et al. (1994). The gene was initially mapped to the long arm of chromosome 6 (6q25.2-q27), a region deleted in some of the AR-JP patients.

In juvenile-onset PD classic symptoms of PD, such as bradykinesia, rigidity, and tremor are present, but it also has its specificities in several aspects. Matsumine et al. (1998) noted that early-onset parkinsonism with diurnal fluctuation (EPDF) is also a dopa-responsive form of parkinsonism and is associated with selective degeneration in the zona compacta of the substantia nigra without Lewy body formation. A distinguishing feature of this phenotype is a benefit from sleep with lessening of parkinsonian symptoms after awakening (Matsumine et al. 1998).

Kitada and co-workers found out that the *PARK2* (*parkin*, also known as E3 ubiquitin protein ligase) gene spans more than 500 kb and has 12 exons. Sasaki et al. suggested that a functional parkin protein may be required for Lewy body formation (Takahashi et al. 1994; Kitada et al. 1998; Matsumine et al. 1998; Sasaki et al. 2004). This protein contains five functional domains, namely the N-terminal ubiquitin-like (UBL) domain, the unique parkin (UP) domain, and the C-terminal RING1, in-between RING (IBR) and RING2 domains. It has been shown that parkin is an E3 ubiquitin ligase that is able to catalyze the addition of ubiquitin molecules to specific substrates, which targets these substrates for degradation *via* the ubiquitin proteasome system (Shimura et al. 2000; Haylett et al. 2011).

Mutations in the *PARK2* gene are those most frequently identified among patients with early-onset of Parkinson's disease (EOPD) (age at onset ≤50 years). They account for up to 50% of autosomal recessive early-onset parkinsonism (AREP) and 15–20% of sporadic EOPD (Kitada et al. 1998). Over 100 types of mutations including sequence substitutions, insertions, and exonic deletions/duplications (or dosage mutations) in the *PARK2* gene have been described in diverse ethnic groups (Hedrich et al. 2004), majority of which are loss-of- function mutations affecting one or more of the functional domains.

Although PD causative mutations of *parkin* are usually homozygous or compound heterozygous, previous studies have hypothesized that the heterozygous mutations may cause haploinsufficiency of the gene and increase the risk for PD (Foroud et al. 2003; Sun et al. 2006; Wang et al. 2010). *Parkin* is particularly susceptible to whole exon deletions and duplications; it is therefore imperative to consider exon dosage mutations when performing mutation screening of *parkin* (Lücking et al. 2000; Periquet et al. 2003). The results of Wang et al. (2010), showed clearly that most (>60%) of the dosage *parkin* mutations identified in both the familial and sporadic EOPD cases were heterozygotes, and that heterozygous dosage mutations alone can increase the overall risk for development of EOPD, especially of AREP

(autosomal-recessive early-onset parkinsonism) subjects ($p = 0.004$). Moreover, the carrier status of dosage mutations has significant impact on the clinical features such as age at onset, characteristics of initial signs, and later complications in both AREP and sporadic EOPD patients. The haploinsufficiency may be caused more by dosage mutations than by point mutations. However, the dosage heterozygotes may not directly lead to PD because there were members in AREP families for dosage heterozygotes who were not affected. Other mutations such as a second point mutation may be needed to constitute a complete genetic contribution to the disease (Wang et al. 2010).

In our study we have analyzed selected exons of *LRKK2* and *PARK2* genes, since in these genes/exons the most frequent pathogenic mutations causing PD have been described.

Materials and Methods

216 PD patients (119 men, 97 women) were included in the study. Written informed consent was obtained from all individuals before participation in the study. All patients were of the same ethnic middle European ancestry. The clinical diagnosis of Parkinson's disease was confirmed by neurologists. Twenty-eight patients (12.9%) reported a positive family history of parkinsonism. The Hoehn–Yahr stage was between 1–5 (mean 2.6). The average age in this cohort was 68 years (35–91), while the mean age at onset (Table 1) was 59.77 years (range 30–86). Thirty-nine patients were defined as the early onset parkinsonism (≤50 years).

All subjects were screened for possible mutations within selected exons in *LRRK2* (exons 31, 35, 41 and 48) and *parkin* (2, 6 and 7) genes using dHPLC (denaturing high-performance liquid chromatography). By this means we also aimed to test for presence of the most common mutations and polymorphism reported in these exons that are responsible for both the early and the late onset form of the disease.

Genomic DNA was isolated from peripheral blood using Gentra Puregene Blood Kit according to the manufacturers' protocol (QIAGEN Sciences, USA). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) containing flanking intronic sequences (Table 2).

Table 1. Distribution of age at onset of the 216 PD subjects

Age at onset (years)	Number of subjects	Percentage
0–29	0	0
30–39	11	5.09
40–49	28	12.97
50–59	56	25.92
60–69	76	35.19
70–79	44	20.37
80–89	1	0.46
Total	216	100

PCR products were generated with 100 ng DNA template in 3 µl 10 × PCR buffer (GeneCraft, Ares Bioscience, Cologne, Germany), 0.15 µl of 10 mmol/l each dNTP (Promega), 0.9 µl of 50 mmol/l MgCl2, 1 µl of 5 µmol/l each primer (Sigma-Genosys, USA) and 1unit BioTherm Taq DNA polymerase (GeneCraft, Ares Bioscience, Cologne, Germany) in a total volume of 30 µl. PCR conditions were: 94°C for 4 min followed by 30 cycles of 94°C for 25 s, the annealing temperatures were 57°C (LRRK2ex31), 59°C (LRRK2ex35, ex48, PARKINex2, ex6, ex7), 60°C (LRRK2ex41) for 25 s, and 72°C for 40 s, with a final extension step of 72°C for 5 min. PCR products of each exonic PCR amplification products were electrophoresed on a 1.5% agarose gel at 150 V for 60 min and visualized by UV illumination staining with SybrGreen I (Invitrogen, Grand Island, NY, USA). SybrGreen I was included in the loading dye and after mixing with the sample final concentration was 6×.

DHPLC analysis was carried out on an automated dHPLC device equipped with a DNA separation column (WAVE Transgenomic, Omaha, NE, USA) according to the manufacturer's protocol. 25 µl of each PCR product (containing 10–100 ng DNA) was denatured at 94°C for 5 min and then gradually reannealed by decreasing the sample temperature from 94°C to 54°C over a period of 30 min. To perform

Table 2. Primer sequences, temperature of annealing, product size (bp) and temperature of DHPLC analyses

Exon	Forward (5'-3")	Reverse (5'-3')	Annealing (°C)	Product size (bp)	T_{DHPLC} (°C) F/R
Lrrk2 ex31	ATGTGAGCAGGCCCAGTTT	AACAAGGAAAGAAAACCCACAA	57	392	56.6/59.2
Lrrk2 ex35	CATTTGCTCAACAAGGTTGG	GCTGCCTTCCAAACAAGTAAA	59	353	55.7/57.3
Lrrk2 ex41	GATGCTTGACATAGTGGACATTT	TGTTTTCCTTTTGACTCTTCTGA	60	351	58.0/59.1
Lrrk2 ex48	GCAATAGTCTAGCTTGTTTAGTTTTCA	TCAGAGGCAGAAAGGAAGAAA	59	377	54.9/56.9
Parkin ex2	TAAGGGCTTCGAGTGATGCT	GCATGAGCAATGGAGCTG	59	272	60.3/62.7
Parkin ex6	ACTGTGGAAACATTTAGAGGAAAAA	GCTCGTGTGGCAGAACAATA	59	297	56.2/59.5
Parkin ex7	GCTGCCTTTCCACACTGAC	TTGTTCTTCTGTTCTTCATTAGCA	59	248	60.6

DHPLC analysis, aliquots of 10 μl of PCR products were injected for 0.1 min on a semiautomated Wave-3500 system (Transgenomic, Omaha, NE, USA).

Analysis took in general approximately 7–8 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of gradient mixture of 0.1 M triethylamine acetate (TEAA) (pH 7.0) with buffer B (0.1 M TEAA and 25% acetonitrile). Temperature for successful resolution of heteroduplex molecules was determined by running fragment-specific melting curves and by using the DHPLC melting algorithm WAVE Maker of the WAVE instrument (Table 2). Data were analyzed using Navigator 1.5.3 software (Transgenomic). Samples with a variation in peak shape when compared to the control samples (DNA from people without PD symptoms) were sequenced with both forward and reverse primers.

Purified amplicons were sequenced in both directions using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, UK) in the forward and reverse directions and resolved on an ABI 3130xl automated sequencer (Applied Biosystems, UK), analysed with DNA Sequencing Analysis v5.2 (Applied Biosystems, UK) and ChromasPro v1.5 software (Technelyisium Pty Ltd., Australia).

Results

In our cohort of 216 early and late onset PD cases we detected one heterozygous missense mutation p.Arg275Trp (R275W, c.823C>T, rs34424986) in exon 7 of the *parkin* (*PARK2*) gene in one patient. In addition, we identified one polymorphism in intron 2 of this gene and seven intronic and one exonic polymorphisms within *LRRK2* gene (Table 3). The polymorphisms identified in *LRRK2* intron 47: IVS47-91insGCCAT, IVS47-91insGCAT and IVS47-20C>T are novel. In order to predict a possible effect of these variants on exon splicing we used the online Human Splicing Finder (version 2.4.1, link: http://www.umd.be/HSF/) software. HSF predicted the IVS47-20C>T variant to create a new potential silencer motif within intron. The IVS47-91insGCCAT and IVS47-91insGCAT create new potential acceptor splice sites.

The DNA analysis of the parents and other family members of the patients carrying the polymorphisms or R275W mutation was not possible, thus no segregation of variants with the disease could be followed.

In the exons 31 and 41 of gene the *LRKK2*, and in exon 6 of the gene *parkin* we did not observe any variant chromatographic profile after DHPLC analyses.

Discussion

In our variable cohort of 216 PD cases we identified in one female patient the presence of a single exon 7 *parkin* (*PARK2*) gene mutation R275W in heterozygous state. This patient had the age at onset 61 years and her first symptom was a tremor on their right hand. Hoehn-Yahr stage was 3. She was administered dopamine agonist MPX 1.2,

Table 3. Polymorphisms found in Slovak population

Allele	Accession N°	Frequency of allele p(A)	Genotypes	*n*
LRRK2 in35: IVS35+23T>A	rs7307276	0.3773	IVS35+23T>A(hetero)	39
			IVS35+23T>A(homo)	62
LRRK2 in47: IVS47-91insGCCAT	novel	0.0278	IVS47-91insGCCAT(hetero)	12
			IVS47-91insGCCAT(homo)	0
LRRK2 in47: IVS47-91insGCAT	novel	0.7315	IVS47-91insGCAT(hetero)	42
			IVS47-91insGCAT(homo)	137
LRRK2 in47: IVS47-90A>G	rs56026228	0.0116	IVS47-90A>G(hetero)	5
			IVS47-90A>G(homo)	0
LRRK2 in47: IVS47-41A>G	rs184962630	0.0023	IVS47-41A>G(hetero)	1
			IVS47-41A>G(homo)	0
LRRK2 in47: IVS47-20C>T	novel	0.0046	IVS47-20C>T(hetero)	2
			IVS47-20C>T(homo)	0
LRRK2 in47: IVS47-9delT	rs11317573	0.5138	IVS47-9delT(hetero)	84
			IVS47-9delT(homo)	69
LRRK2 ex48: 7155A>G	rs33962975	0.0926	7155A>G(hetero)	38
			7155A>G(homo)	1
parkin ex2: IVS2+25T>C	rs111356273	0.1829	IVS2+25T>C(hetero)	75
			IVS2+25T>C(homo)	2

n, the number of subjects carrying this genotype (among 216 pacients).

monoamine oxidase inhibitor (MAO-B) Azilect, and she showes no fluctuations. In intron 2 of the *parking* gene also the presence of known polymorphism was identified.

No mutations of the *LRRK2* gene were found in tested group. However, the presence of seven intronic and one exonic *LRRK2* variant was observed, all within intron 47.

The only mutation identified in our cohort, R275W, affects the RING1 domain of parkin and it is one of the most frequent amino acid change within this gene, confirming previous reports from other European studies (Abbas et al. 1999; Hedrich et al. 2004; von Coelln et al. 2004; Sironi et al. 2008).

Sriram and co-workers suggested that the R275W mutation could impair the ubiquitin-proteasome system through sequestration into aggresome-like structures in the cell and away from their site of normal function (West et al. 2002; Sriram et al. 2005).

Drosophila model overexpressing R275W mutation was found to exhibit dopaminergic degeneration and mitochondrial abnormalities similar to *parkin* knockout flies, including mitochondria with large vacuoles, concentric membranous structures, disorganized cristae, or degenerated membranes. These studies suggest that parkin plays a key role in maintaining mitochondrial stability in certain cell types (Wang et al. 2007).

Two mutant isoforms, R256C and R275W, within RING finger 1 of the Parkin protein (238–293 amino acids), produced an unusual distribution of the protein, with large cytoplasmic and nuclear inclusions. Cookson et al. (2003) replicated this observation in primary cultured neurons and demonstrate, by the accumulation/co-localization of cytoskeletal protein vimentin, that the inclusion bodies are aggresomes, a cellular response to misfolded protein.

The range of the so far reported *parkin* mutations varies from point mutations to complex rearrangements including deletions and/or multiplications of complete exons. Oliveira et al. found the RING1 mutations primarly in heterozygous PD patients with a much later age at onset (mean AAO: 49.2 years) but were not found in controls in their study or several previous reports. These findings suggest that mutations in *parkin* contribute to the common form of PD and that heterozygous mutations, especially those lying in exon 7, act as susceptibility alleles for late-onset form of Parkinson disease (Oliveira et al. 2003). Also our patient, carrier of R275W, has the age at onset rather high – 61 years.

In the study of Sironi et al. (2008), the father of two affected siblings developed a classical late onset PD. He was a carrier of R275W.

Several other studies suggest that a single *parkin* mutation may cause EOPD or represent a risk factor for late-onset PD (Foroud et al 2003; Bertoli-Avella et al. 2005; Sun et al. 2006; Deng et al. 2008). The association of *parkin* haploinsufficiency in sporadic Parkinson's disease further implicates the role of parkin in the more common form of the disease.

However, pathogenicities of *parkin* mutations, especially those in heterozygotes, remain controversial, possibly due to confusions caused by the "mixed" effects of all types of mutations (Wang et al. 2010).

The single heterozygous state might exert haploinsufficiency effects rather than dominant negative effects. By contrast, some missense mutations might have a dominant negative effect as missense mutations in functional domains (Ubl or RINGS), resulting in an earlier onset than with mutations in other regions (Lohmann et al. 2003; Hattori and Mizuno 2004).

In order to evaluate the role of heterozygous mutations in PD, as observed in our patient, it is necessary to compare their frequencies in patients and controls. Brüggemann and co-workers demonstrated that a clinical follow-up investigation of mutation carriers is important to assess the mutation frequency in truly unaffected individuals. While the implications of heterozygous mutations in PD cannot be conclusively determined, previous data point to a role as a susceptibility factor that is able to cause at least subtle signs of PD later in life. Therefore, they suggested to perform further prospective studies in older controls, in conjunction with a careful neurological examination, in order to elucidate the clinical relevant question of how many of them will eventually develop PD (Brüggemann et al. 2009). Thus, the control study requires large control group and long follow up of potentially identified mutation carriers, which was not possible in our pilotal study.

In the few patients in whom only a single heterozygous *parkin* mutation was detected, it is possible that a second mutation escaped identification by the methods employed, or that some mutations in heterozygous forms are sufficient to cause disease (Klein et al. 2000).

Interestingly, heterozygous mutations in other autosomal recessive PD genes have also been associated with the development of PD, most clearly for carriers of a heterozygous *PINK1* mutation (Abou-Sleiman et al. 2006; Hedrich et al. 2006). Such heterozygous mutations in recessive PD genes may modify the phenotype in patients with mutations in other PD genes (Klein et al. 2007; Funayama et al. 2008).

Several mutations rather frequent in different populations were not present in our cohort. One example is R1441G associated with PD in the Basque region (Gorostidi et al. 2009, Mata et al. 2009a). Similarly, no carriers of R1441G, R1441C, or R1441H mutations were found among our cases.

The *LRRK2* G2019S mutation is the most frequent known cause of familial and sporadic PD. A heterogeneous distribution was observed with high frequencies in North African Arab countries, the Middle East, southern Europe, North American Ashkenazi Jewish populations and in South American countries with known European ethnic influence.

Frequencies ranged from the no cases to 35.7% in sporadic and 42% in familial North-African Arab patients (Brás et al. 2005; Lesage et al. 2005, 2006, 2008; Clark et al. 2006; Deng et al. 2006; Gaig et al. 2006; Goldwurm et al. 2006; Infante et al. 2006; Ozelius et al. 2006; Marongiu et al. 2006; Mata et al. 2006, 2009b; Civitelli et al. 2007; Cossu et al. 2007; Ferreira et al. 2007; González-Fernández et al. 2007; Ishihara et al. 2007; Orr-Urtreger et al. 2007; Perez-Pastene et al. 2007; Squillaro et al. 2007; Hulihan et al. 2008; Munhoz et al. 2008; Pimentel et al. 2008; Correia Guedes et al. 2009; De Rosa et al. 2009; Floris et al. 2009; Gorostidi et al. 2009).

There were not found any known pathogenic mutations (G2019S or I2020T) within exon 41 of *LRRK2* gene in the studied patients. This result of the case doesn´t differ from the studies which were made with other European populations: Austria, Poland, Holland, Belgium, or from Africa: Niger, or from Asia: Taiwan, Singapore, South Korea (Calne et al. 1992; Bialecka et al. 2005; Lu et al. 2005; Mata et al. 2005; Tan et al. 2005; Fung et al. 2006; Farrer et al. 2007; Cho et al. 2007; Haubenberger et al. 2007; Choi et al. 2008; Lin et al. 2008; Nuytemans et al. 2008; Okubadejo et al. 2008; Macedo et al. 2009).

First of the polymorphisms identified in our cohort, IVS35+23T>A in *LRRK2,* was described first time by Schlitter et al. 2006 in a German cohort of 120 patients (Schlitter et al. 2006). The pathogenic role is unknown. Frequency in our group of patients was 38%.

LRRK2 variant IVS47-41G>A was found in 0.8% frequency in a 60 PD families and wasn't present in the control sample (Di Fonzo et al. 2006a). However, the authors descibed that the IVS47-41G>A doesn't cosegregate with the disease (Di Fonzo et al. 2006b). In our group this polymorphism was present only in 0.2%. The patient carrying this variant had rigidity of muscles, age at onset was 63. He takes levodopa (Stalevo 600) and amantidin without fluctuations. The Hoehn-Yahr score was 3.

Two groups desribed the intronic variant IVS47-9delT of *LRRK2* as non pathogenic (Di Fonzo et al. 2006b, Shojaee et al. 2009). In our cohort it was present at frequency of 51%.

This silent point mutation (c.7155A>G, p.Gly2385Gly) in exon 48 of the *LRRK2* gene is located in the WD40 domain and change the codon from GGA to GGG. Mata et al. and Di Fonzo et al. found this variant with similar frequency in cases and controls, therefore this change was considered as neutral (Mata et al. 2005; Di Fonzo et al. 2006a). The frequency was 9% of this allele in our group of patients.

The *parkin* intron 2 variant IVS2+25T>C was identified by Abbas et al. (1999). They found this change also in control subjects and have concluded that this change is not sufficient to cause PD by themselves in European population. In our group of patients was the frequency of this allele 19%.

We identified also novel polymorphisms with possible role in the pathogenesis of PD. Two patients carried the IVS47-20C>T polymorphism within intron 47 of *LRRK2*. The first patient had age at onset 56 years. Her first symptom was tremor on their right hand, she had Hoehn-Yahr score 3. She takes MAO B inhibitor (Azilect) and dopamine agonist (MPX 1.2) without fluctuations. The second patient with this variant was 71 years old when the first symptoms (tremor on their right hand) appeared. The Hoehn-Yahr score was 2.5. He use levodopa (Stalevo 600) without fluctuation, but he had mild dementia (treated with Exelon). As the Human splicing finder indicates (http://www.umd.be/HSF/), this variation may create new potential silencer regulatory element that might negatively affect correct exon splicing.

LRRK2 variants IVS47-91insGCCAT and *LRRK2* IVS47-91insGCAT were present in cca. 88% of patients in our group. The *in silico* HSF analysis showed that these intronic variants appear to significantly modify the recognition of natural splice site. They can create a potential new acceptor splice sites. However, further studies employing patients and controls' splice product are required in order to test for the effect on splicing of these 3 novel variants.

It can be concluded that the point mutations in exons 31, 35, 41, 48 of *LRRK2* gene and in exons 2, 6, 7 of *parkin* causes rarely Parkinson disease in Slovak republic, which corroborates data from other studies from countries of Central/Eastern Europe.

However, we could not completely exclude the possibility that the patients carry other mutations or rearrangements within tested genes, because no molecular analysis was performed of all exons of the *LRRK2* and *PARK2* genes. In the follow up of this study also a gene dosage studies, especially of *PARK2* gene have to be considered, in order to uncover possible larger genomic deletions. We also plan to perform the genetic screening of other genes (e.g. SNCA, PINK1, DJ-1) possibly involed in the pathogenesis of PD in Slovak patients.

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