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Exon dosage variations in Brazilian patients with Parkinson's disease: Analysis of *SNCA*, *PARKIN*, *PINK1* and *DJ-1* genes

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Abstract. Parkinson's disease is one of the most common neurodegenerative disorders associated with aging, reaching $\sim 2\%$ of individuals over 65 years. Knowledge achieved in the last decade about the genetic basis of Parkinson's disease clearly shows that genetic factors play an important role in the etiology of this disorder. Exon dosage variations account for a high proportion of Parkinson's disease mutations, mainly for *PARKIN* gene. In the present study, we screened genomic rearrangements in *SNCA*, *PARKIN*, *PINK1* and *DJ-1* genes in 102 Brazilian Parkinson's disease patients with early onset (age of onset $\leqslant$ 50 years), using the multiplex ligation-dependent probe amplification method. Family history was reported by 24 patients, while 78 were sporadic cases. Screening of exon dosage revealed *PARKIN* and *PINK1* copy number variations, but no dosage alteration was found in *SNCA* and *DJ-1* genes. Most of the carriers harbor heterozygous deletions or duplications in the *PARKIN* gene and only one patient was found to have a deletion in *PINK1* exon 1. Data about dosage changes are scarce in the Brazilian population, which stresses the importance of including exon dosage analysis in Parkinson's disease genetic studies.

Keywords: Copy number variation, early-onset, MLPA, Deletion, Duplication

1. Introduction

Parkinson's disease (PD) is mainly characterized by a progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* and also by the involvement of other *nuclei* in the brain. PD is the second most common neurodegenerative disorder after Alzheimer's disease, affecting $\sim 2\%$ of individuals over 65 years [7] and occurring worldwide. The clinical symptoms vary, but they are mainly characterized by rest tremor, postural instability, rigidity, and bradykinesia, as well as, a good response to levodopa therapy [8]. Both genetic and environmental factors can be implicated in its etiology. In the past decade, several genetic factors have been identified and five genes were conclusively implicated as causative of autosomal dominant (*SNCA* and *LRRK2*) or autosomal recessive (*PARKIN*, *PINK1* and *DJ-1*) forms of PD [11].

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Besides missense mutations and single nucleotide polymorphisms, genomic rearrangements (deletions and multiplications containing entire exons or genes) are common mutations found in PD patients [21]. This type of alteration plays a particularly important role in the *PARKIN* gene, where they are significantly more frequent [21]. Point mutations and copy number alterations have been described in all 12 exons of *PARKIN* and they typically leads to juvenile-onset (< 21 years) or early-onset forms of PD ($\leqslant 50$ years) [1,19]. Exon rearrangements account for more than 50% of patients with *PARKIN* mutations [13], which highlight the importance of screening copy number variations in this gene. These mutations can be present in homozygous, compound heterozygous or heterozygous state, suggesting that *PARKIN* may present itself as a causative or a susceptibility genetic factor [10,26].

Additionally to *PARKIN*, whole gene multiplications (duplications or triplications) have been described for *SNCA* gene, which leads to overexpression of the protein caused by the extra copies of the gene. *SNCA* is the only PD gene that undergoes duplications of its entire extension, which makes its gain-of-function pathogenic process unique between the genetic causes of PD [21]. In addition, there is a direct correlation between the dose of *SNCA* multiplications (duplication or triplication) with the severity and the age of onset of the patient's symptoms. For this reason, *SNCA* dosage alteration is one of the few well- established genotype-phenotype correlations in PD.

Mutations of exon dosage have also been identified in two additional PD genes, *PINK1* [20] and *DJ-1* [9], but they were observed in a relative lower frequency than those seen in *PARKIN*. No copy number variation affecting the dosage of *LRRK2* or its exons was ever found [21].

In this study, we present the first analysis of genomic rearrangements in known parkinsonian genes in Brazilian PD patients, using the multiplex ligation-dependent probe amplification method (MLPA), to ascertain whether exon dosage changes in *SNCA*, *PARKIN*, *PINK1* and *DJ-1* are an important genetic factor in the etiology of this disorder in our population.

2. Materials and methods

The molecular analysis was performed in a sample of 102 unrelated Brazilian patients (64 males and 38 females; mean age: 51.9 ± 10.4 years) diagnosed with early-onset PD ($\leqslant 50$ years), including two cases of juvenile parkinsonism (age at onset of 12 and 14 years). The mean age at onset (AAO) of our sample was 41.4 $\pm$ 6.6 years. The patients were recruited by specialized clinicians from movement disorder clinics of major public hospitals from the Southeast and Midwest regions of Brazil and fulfilled criteria established for the clinical diagnosis of PD [14]. Family history of PD was self-reported by 24 patients, while 78 were sporadic cases. No individual reported an Ashkenazi Jewish background. The research protocol was approved by the Institutional Ethics Committee (REG. 032.2.2008), and written informed consent was obtained from all subjects.

The DNA of each participant was obtained from a sample of peripheral blood through extraction using Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare). Dosage analysis was performed by MLPA. The commercially available kit P051-C1 (MRC Holland) was used for the multiplex dosage of exons in genes *PARKIN*, *SNCA*, *DJ-1* and *PINK1*. MLPA reaction conditions followed the protocol suggested by MRC Holland, except for the quantity of DNA used (50 ηg) and the time of the initial denaturation (40 minutes) to fix problems with incomplete denaturation in some samples. MLPA fragments were analyzed on a 3130 Genetic Analyzer (Applied Biosystems) following the configuration recommended by MRC Holland. The results were processed on GeneMapper software v3.7 (Applied Biosystems) and then analyzed on Coffalyser (http://old.mlpa.com/coffalyser/index.html), a macro written in Visual Basic that runs within Microsoft Office Excel. The peak areas were normalized against the sum of the areas observed in the control probes for each patient. The normalized value was compared with the mean normalized value of a group of control to generate a ratio that represents the relative number of copies. A value of one or near one is normal (two copies), values below 0.65 point a deletion, 1.35–1.65 means three copies and so on.

All abnormal findings pointing to possible deletions and duplications detected by MLPA were verified in a second independent experiment and, after, in a quantitative PCR assay (qPCR). The dosage by qPCR was performed in a 7500 Real-Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). The relative copy number was calculated through a $\Delta\Delta$CT method, using ALB as an internal reference as previously described [5]. False positives were sequenced to verify the existence of SNPs within the sequence of hybridization of the MLPA probe and patients who exhibited dosage changes

Table 1
Duplications and deletions observed in Brazilian early onset PD patients

Patient	Region	Variation	AAO	Family history[a]	M/F[b]
2083	*PINK1* – Exon 1	Deletion	45	+	M
2099	*PARKIN* – Exon 4	Deletion	12	–	M
2256	*PARKIN* – Exon 4	Duplication	37	–	F
2396	*PARKIN* – Exon 5–6	Deletion	46	+	M
	PARKIN – Exon 3	Duplication			

AAO age at onset.
[a] – absent; + present.
[b] M male; F female.

had the entire exons of the respective gene sequenced in order to screen for a possible second pathogenic mutation. Sequencing was performed on a 3130 Genetic Analyzer (Applied Biosystems) with the BigDye Terminator v3.1 (Applied Biosystems). Primer sequences are available upon request.

3. Results

Screening of the exon dosage through MLPA in 100 Brazilian patients with early onset PD and two probands with juvenile onset, revealed four patients (3.9%), two familial and two sporadic cases, with copy number variations of specific exons in genes *PARKIN* and *PINK1*. MLPA Kit P051-C1 tests for all 12 *PARKIN* exons and multiplications were detected in exons 3 and 4 (three copies). Deletions were detected in exons 4, 5 and 6 (one copy). In *PINK1*, all 8 exons were analyzed, but only exon 1 was found to be deleted (one copy) in one patient. No duplications were found in *PINK1*. In addition, no *SNCA* gene multiplication or *DJ-1* dosage alterations were observed in our sample (Table 1). Most of our cases consisted in heterozygous deletions or duplications in the *PARKIN* gene (patients 2099 and 2256). One patient, 2396, harbors a deletion of *PARKIN* exons 5-6 and a duplication of *PARKIN* exon 3 (Fig. 1).

The MLPA screening detected a heterozygous deletion of *PINK1* exon 1 in 6 patients. However, only one *PINK1* exon 1 deletion, observed in patient 2083, was confirmed after validation by real time PCR, revealing a high occurrence of false positives in this region. Sequencing revealed that the false positives were not due to SNPs and it is postulated that it may be as a result of incomplete denaturation of this particular probe which occurs in a GC-rich region.

No point mutations within the exonic sequence of the affected genes (*PARKIN* and *PINK1*) were detected in the patients with heterozygous variants, with the exception of patient 2256, who carries a duplication in *PARKIN* exon 4 and also harbors the silent variant c.1021C>T in *PARKIN* exon 9 (p.L341L).

4. Discussion

Since exonic dosage alterations in the *PARKIN* gene were first identified in families with juvenile parkinsonism, segregating as an autosomal recessive form of PD [17], copy number variations of exons or entire genes related to PD have been gaining an increased attention over the years and have been screened in several populations across the world [21]. However, data about these mutations are scarce in the Brazilian population. Only one study has analyzed genomic dosage in *PARKIN*, *SNCA*, *DJ-1* and *PINK1* in a sample of 45 Brazilian PD patients with early onset [4].

The screening of quantitative changes in *PARKIN*, *PINK1*, *DJ-1* and *SNCA* genes in our sample revealed five copy number variations in four probands. Among the changes identified, *PARKIN* was the most frequently mutated, harboring four copy number alterations located in exons 3, 4, 5 and 6. Exon 4 was the most affected region, accounting for 50% of the total mutations in *PARKIN*, corroborating the data present in the literature [12,13,23,27].

Among all the changes identified in *PARKIN* so far, exon rearrangements are responsible for, approximately, 50% of cases [13]. Because of its genomic structure, the *PARKIN* gene is prone to a high rate of irregular meiotic recombination processes that lead to exonic rearrangements [2]. These dosage mutations can be present in either homozygous, compound heterozygous or heterozygous states. We identified a heterozygous deletion in exon 4 of *PARKIN* gene in one patient who has a juvenile AAO of 12 years and no family history of the disease. This alteration had already been reported by other groups in PD patients and healthy individuals [3,6,15,22]. Kay and colleagues (2010) demonstrated that heterozygous dosage mutations in exons 2–4 are common and well-tolerated in control subjects [15]. However, it is possible that harboring a single *PARKIN* mutation could be a risk factor that, combined with other unknown genetic and environmental factors, can lead to PD phenotype. We have also found an exon

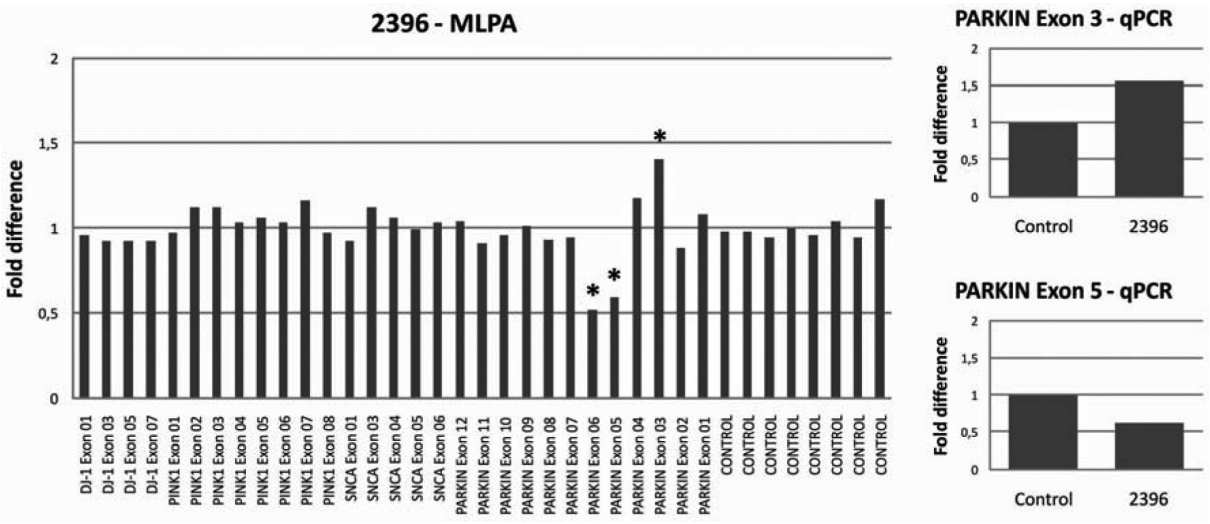

Fig. 1. Left: MLPA analysis result of a patient (2396) that harbors two mutations in *PARKIN* gene: duplication in exon 3 (3 copies) and a deletion of exons 5–6 (1 copy). Asterisks indicate the probes that were affected by the genomic rearrangements in the three exons of *PARKIN*. The vertical axis represents the ratios calculated and the horizontal axis represents the different probes within the SALSA P051 MLPA kit. Right: quantitative PCR validation of the duplication in exon 3 (upper) and of the deletion in exon 5 (lower).

4 duplication in another sporadic case that manifested the symptoms of the disease at 37 years of age.

An exon 5–6 deletion was detected in a male PD patient. Deletions in exon 5 were previously found in PD patients by several other studies [21], and it was also reported to be present in a healthy female [18]. Deletions in exon 6, however, were never detected in healthy individuals. Moreover, the patient also harbors a duplication in exon 3. He manifested PD symptoms at 46 years and reported a positive family history of parkinsonism, different from the other probands of our sample with mutations in *PARKIN* that were all sporadic cases of the disease. We believe that segregation studies would help clarify the pathogenic value of both variants, however, biological material of family members is not available at this time.

In the analysis of the *PINK1* gene, the MLPA technique initially detected an apparent heterozygous deletion of exon 1 in six patients. However, by verification through qPCR, the deletion was not confirmed in five cases. Only patient 2083, a case of familial PD, with AAO of 45 years, was found to harbor a novel exon 1 deletion. Only deletions in this gene were reported associated with PD so far and, in exception of one case of a complete deletion of *PINK1* [20], no rearrangement was observed affecting exons 1 or 2 [21]. To our knowledge, this is the first report of a patient with a deletion affecting only exon 1 of *PINK1*.

SNCA multiplications have been implicated in autosomal dominant forms of PD since 2003 [25]. However, it became clear that whole-gene multiplications in *SNCA* locus are a rare form of parkinsonism and may account for only a small fraction of the total number of PD patients [21,24]. Corroborating this data, we have not found any dosage alteration involving the *SNCA* gene and the *DJ-1* gene in our sample. The *DJ-1* gene is another example of a rare genetic factor associated with PD. Point mutations and copy number variations in this gene represent less than 1% of all genetic variants found in PD [21].

In this study, we have used MLPA to screen for exon deletions and duplications, which showed to be effective and reproducible, but it is necessary to confirm positive results by real time PCR, as false positives can arise. In conclusion, dosage mutations are relatively common in Brazilian early onset PD patients. The frequency observed by our study was similar to others from the literature [6,13,16]. *PARKIN* presented the majority of dosage alterations compared with the other known PD genes, as expected, while *PINK1*, *SNCA* and *DJ-1* exon rearrangements were less common. Our results confirm the importance of including exon dosage analysis in PD genetic studies, particularly in *PARKIN* gene, for a better understanding of PD etiology.

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