Rapid and Reliable Detection of Exon Rearrangements in Various Movement Disorders Genes by Multiplex Ligation-Dependent Probe Amplification

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Abstract: Because of the occurrence of different types of mutations, comprehensive genetic testing for Parkinson’s disease (PD), dopa-responsive dystonia (DRD), and myoclonus-dystonia (M-D) should include screening for small sequence changes and for large exonic rearrangements in disease-associated genes. In diagnostic and research settings, the latter is frequently omitted or performed by laborious and expensive quantitative real-time PCR (qPCR). Our study aimed to evaluate the utility of a novel method, multiplex ligation-dependent probe amplification (MLPA), in molecular diagnostics of movement disorders. We have analyzed, by MLPA, genomic DNA from 21 patients affected with PD, DRD, or M-D, in which the presence of exon rearrangement(s) (n = 20) or of a specific point mutation (detectable by MLPA, n = 1) had been established previously by qPCR or sequencing. In parallel, we have studied, in a blinded fashion, DNA from 49 patients with an unknown mutational status. Exon rearrangements were evident in 20 samples with previously established mutations; in the 21st sample the known specific point mutation was detected. We conclude that MLPA represents a reliable method for large-scale and cost-effective gene dosage screening of various movement disorders genes. This finding reaches far beyond a simple technical advancement and has two major implications: (1) By improving the availability of comprehensive genetic testing, it supports clinicians in the establishment of a genetically defined diagnosis; (2) By enabling gene dosage testing of several genes simultaneously, it significantly facilitates the mutational analysis of large patient and control populations and thereby constitutes the prerequisite for meaningful phenotype–genotype correlations. © 2007 Movement Disorder Society

Key words: multiplex ligation-dependent probe amplification; Parkinson’s disease; dopa-responsive dystonia; myoclonus-dystonia; exon rearrangements.

Parkinson’s disease (PD), dopa-responsive dystonia (DRD), and myoclonus-dystonia (M-D) represent a group of movement disorders with partial clinical overlap, whose hereditary forms have been associated with various mutations in several responsible genes. To date, changes in five genes, namely, α-synuclein (SNCA; PARK1, MIM*163890, no. 168601), Parkin (PARK2, MIM*602544, no. 600116), Pten-induced putative kinase 1 (PINK1; PARK6, MIM*608309, 605909), DJ-1 (PARK7, MIM*602533, 606324), and Leucine-rich re-
peat kinase 2 (LRRK2; PARK8, MIM*609007, no. 606324), have been definitely implicated in the pathogenesis of PD (for review, see Refs. 1 and 2). In most cases, DRD is caused by mutations in the GTP cyclohydrolase I (GCH1; MIM*600225, no. 128230) gene, and in a smaller fraction by mutations in the Tyrosine hydroxylase (TH; MIM*191290, no. 605407) gene. Finally, mutations in the e-Sarcoglycan gene (SGCE; MIM*604149, no. 159900) have been associated with M-D.4

In most of the aforementioned genes, both small sequence changes (point mutations, small deletions/insertions) and large exonic rearrangements (deletions/multiplications of whole exon(s)) have been described.5-10 The latter play an important role in the Parkin, SNCA, GCH1, and SGCE genes. For instance, ~55% of patients with mutations in Parkin carry exon rearrangements,11 which stresses the importance for genetic testing.

Since exon rearrangements cannot be detected by qualitative mutation-screening methods (e.g. SSCP, dHPLC, sequencing), they are often not tested for or are assessed using the accurate but laborious and expensive quantitative real-time PCR (qPCR) analysis.5

In clinical practice, physicians are increasingly faced with commercially available genetic tests for monogenic movement disorders that currently include screening for rearrangements in Parkin only. Further, large-scale testing for exon rearrangements should also be performed in research studies aiming to establish phenotype–genotype correlations.

Recently, a new method, multiplex ligation-dependent probe amplification (MLPA), has been developed for rapid detection of exonic deletions and multiplications, and specific recurrent mutations12 and has become commercially available for testing of disease genes for a variety of disorders.13-15

MLPA is based on amplification of the probe, generated by ligating two adjacent oligonucleotides. As a precondition, two oligonucleotides have to hybridize completely to the target sequence in genomic DNA. Consequently, an absence or increased/decreased number of sequences complementary to the probe will result in no probe amplification or its increased/decreased amplification. Likewise, oligonucleotides will not be ligated, and thus the probe will not be amplified if the first oligonucleotide has a mismatch at the 3′ nucleotide when annealed to the target sequence.12 In a single MLPA reaction, up to 45 different probes can be amplified simultaneously, allowing for the detection of an aberrant copy number of up to 45 genomic sequences (e.g. exons of the specific genes).

Using a qPCR assay with hybridization probes (LightCycler, Roche Diagnostics, Mannheim, Germany), the presence of exonic rearrangements had previously been detected in 20 patients suffering from the aforementioned movement disorders. Additionally, by sequencing, we identified the recurrent p.G2019S mutation in LRRK2 in another PD patient. In search for an equally reliable, and yet more feasible means of detecting these mutations, we used these samples with a known mutational status to evaluate the utility of MLPA.

PATIENTS AND METHODS

Study Design

Using multiplex ligation-dependent probe amplification (MLPA), we have analyzed genomic DNA from 21 patients (16 PD, 3 DRD, and 2 M-D cases) with known Parkin, LRRK2, GCH1, and SGCE mutations and from 49 patients (34 PD, 8 DRD, and 7 M-D cases) with an unknown mutational status. The existence of exon deletions or multiplications in 20 of the investigated samples had been identified previously by qPCR. The presence of the recurrent point mutation p.G2019S in LRRK2 in one sample had been demonstrated by sequencing. The qPCR results for 9 patients were newly established and the remainder has been reported elsewhere.5,6,11,16-18 Fresh blood samples from 3 previously reported patients were obtained since the initial report.

The study design was as follows: the MLPA reaction and subsequent electrophoresis were performed for 12 samples in parallel. One investigator (K.H.) was aware of the mutational status of all samples and chose seven with unknown mutational status and three mutation-positive samples for each run. Blind to the mutational and clinical status of these 10 samples, three investigators (A.D., M.G., and A.G.) conducted the MLPA analysis and also the results. In addition, in every run, the same two mutation-negative samples were included as a reference for copy number quantification.

MLPA Analysis

For MLPA analysis we have used two sets of standard, commercially available probes (SALSA P051 and P052 Parkinson MLPA kits, MRC Holland, Amsterdam, The Netherlands; [http://www.mrc-holland.com/pages/p051_p052pag.html]) and a third set enriched with novel SGCE test probes (SALSA P099 kit special version: GCH1-TH-SGCE). Probes for the same exon of one gene in different kits were different. A list of the probes contained in these three kits is given in Table 1.

Experimental procedures were conducted according to the manufacturer’s protocol. Amplification products
were visualized after PAGE on an automated sequencing machine (LI-COR; Lincoln, NE).

For each sample, the analysis yielded a pattern of bands of different sizes and intensities (Fig. 1), each representing one amplified probe from the kit used. The acquired image was saved as a Tag Image File Format (TIFF), and the volume (the surface multiplied by the intensity) of each band was transformed into the curve, and the area under the curve was translated into a numerical value using the TotalLab software (Nonlinear Dynamics; Newcastle upon Tyne, UK). All values were normalized employing a modified “nearest neighbor” model, in which the value of each band was divided by the sum of volumes of the four nearest, appropriate bands (done in Microsoft Excel). Often, several exons of one gene are deleted. Therefore, none of the four divisor bands used belonged to the same gene as the one being normalized. Once normalized, the volume of each band was divided by the average normalized value of the corresponding bands of the two mutation-negative standard controls. Result thresholds widely used in other quantification studies were applied5,19,20: ratios between 0.38 to 0.74 (homozygous deletion) (Table 2). Increased ratios varied between 1.38 and 1.49 (heterozygous duplication), with an exception of one value that was 1.82 (homozygous duplication or triplication) (Table 2). Increased/decreased ratios of “nearest-neighbor” probes to those indicating deletions/multiplications were observed (Fig. 1) and considered to be an artifact caused by the normalization process (e.g. a normal value gets higher when divided by a value lower than average). Differentiation between normalization artifacts and true results were made after visual inspection of intensity of bands on the gel (Fig. 1).

In the 21st investigated sample (L-402, Fig. 1), we detected an additional band corresponding to the specific probe for the p.G2019S mutation in LRRK2. When the MLPA results were compared with those obtained with qPCR, the previously established mutational status was confirmed for all samples, except for T-1 and L-324.

**RESULTS**

In 20 of the investigated samples we detected at least one band, corresponding to a Parkin, GCH1, or SGCE exon with an aberrant normalized value ratio. Decreased ratios were either 0.0 (homozygous deletion) or ranged from 0.38 to 0.74 (heterozygous deletion) (Table 2). Increased ratios varied between 1.38 and 1.49 (heterozygous duplication), with an exception of one value that was 1.82 (homozygous duplication or triplication) (Table 2). Increased/decreased ratios of “nearest-neighbor” probes to those indicating deletions/multiplications were observed (Fig. 1) and considered to be an artifact caused by the normalization process (e.g. a normal value gets higher when divided by a value lower than average). Differentiation between normalization artifacts and true results were made after visual inspection of intensity of bands on the gel (Fig. 1).

![FIG. 1. MLPA analysis results of four mutation-positive DNA samples (T-1, L-389, L-402, and L-1523). Gel images depicting pattern of bands obtained by MLPA analysis with the P052 (left) and P051 (right) kits are shown on the margins of the figure. Histograms that present all values of normalized ratios between the probe-specific band volume and the average value of the corresponding bands of the two mutation-negative controls are given in the middle. Each band with an altered intensity is boxed together with two adjacent unaltered bands (boxes a–g), and the corresponding ratio change is marked on one of the histograms. Because of the normalization process, artificially increased/decreased ratios of nearest-neighbor probes, to those indicating deletions/multiplications, are evident. An asterisk marks the position of the band apparent only if a sample carries the p.G2019S mutation in LRRK2 (as L-402 does).](image-url)
### TABLE 2. Results of the MLPA analysis

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Kit number</th>
<th>exon deleted/duplicated exons, as assessed by qPCR</th>
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<tbody>
<tr>
<td></td>
<td>P051</td>
<td></td>
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<tr>
<td>T-1</td>
<td>P051</td>
<td>1.00 1.03 0.56 0.94 1.02 0.98 1.03 1.01 0.52 0.95 1.04 0.99 1.01 1.07 0.45 1.14 1.03 1.02 0.94 1.08 0.98 1.05 1.02 0.88</td>
</tr>
<tr>
<td>T-2</td>
<td></td>
<td>1.05 1.01 1.10 1.12 0.45 0.97 1.01 0.96 0.96 1.01 1.02 1.01 0.96 0.95 1.12 1.11 0.56 1.00 0.97 0.99 1.04 1.10 1.13 1.00</td>
</tr>
<tr>
<td>T-44</td>
<td></td>
<td>0.97 0.60 0.63 0.60 0.56 0.99 0.99 1.05 1.18 0.88 1.00 0.88 0.94 0.74 0.70 0.70 0.45 0.58 1.02 0.97 1.02 1.17 0.98 1.06 1.00</td>
</tr>
<tr>
<td>B-12</td>
<td></td>
<td>0.99 0.96 1.05 1.06 0.92 0.94 0.49 0.90 0.93 0.99 0.88 0.99 1.03 0.98 0.98 0.97 0.99 0.50 0.94 1.03 1.00 1.07 0.91</td>
</tr>
<tr>
<td>B-15</td>
<td></td>
<td>1.06 0.94 1.10 1.17 0.97 0.96 0.51 0.95 1.00 1.05 0.89 0.96 1.06 1.04 0.99 0.95 1.09 0.96 0.49 0.99 1.07 1.03 1.00 0.93</td>
</tr>
<tr>
<td>B-151</td>
<td></td>
<td>0.91 0.49 1.05 1.15 0.59 0.96 1.05 0.97 0.92 1.09 1.02 1.03 0.91 0.59 0.96 0.83 0.54 0.96 1.02 1.04 0.94 1.03 0.93 0.86</td>
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<td>L-324</td>
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<td>0.90 1.02 0.52 0.58 1.06 1.08 1.32 1.40 1.32 1.39 1.48 1.34 0.89 1.12 0.45 0.42 1.04 0.98 1.19 1.38 1.46 1.43 1.38 1.31</td>
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<tr>
<td>L-359</td>
<td></td>
<td>0.99 1.09 0.90 0.56 1.09 0.99 1.04 1.04 1.00 0.89 1.04 0.98 0.92 0.94 0.99 0.46 0.99 0.94 1.01 0.88 1.10 0.98 1.06 0.90</td>
</tr>
<tr>
<td>L-387</td>
<td></td>
<td>0.98 0.55 0.57 0.57 0.51 1.00 1.05 1.10 1.09 0.89 1.04 0.90 1.00 0.55 0.52 0.47 0.47 0.92 0.88 0.91 0.89 1.06 1.07 0.81</td>
</tr>
<tr>
<td>L-389</td>
<td></td>
<td>1.02 1.82 1.03 0.63 0.47 0.94 1.02 1.10 0.98 0.97 0.98 0.94 1.01 1.82 0.91 0.46 0.43 1.06 0.94 1.02 0.95 1.00 1.04 0.84</td>
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<tr>
<td>L-561</td>
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<td>0.02 1.00 1.03 0.61 1.04 0.98 0.97 1.00 0.92 0.96 0.97 0.96 0.99 1.06 0.88 0.38 1.00 1.00 1.04 1.10 1.03 0.99 0.78 1.04</td>
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<tr>
<td>L-1523</td>
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<td>1.08 0.88 0.96 1.00 1.04 0.96 0.00 0.00 0.00 1.00 1.04 1.12 1.05 0.98 1.00 0.86 0.98 0.92 0.00 0.02 0.00 1.00 1.02 0.94</td>
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<tr>
<td>L-2534</td>
<td></td>
<td>1.03 1.07 1.01 1.03 1.05 0.98 1.00 0.58 0.56 1.01 1.07 1.03 0.98 0.99 0.94 0.95 1.03 1.02 1.01 0.57 0.46 1.02 0.96 1.04</td>
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<td>L-2536</td>
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<td>1.02 1.15 0.69 0.68 1.00 1.02 1.03 0.60 0.51 1.05 1.03 1.03 1.00 0.99 0.48 0.48 1.00 1.03 0.99 0.57 0.51 1.05 0.97 1.03</td>
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<tr>
<td>L-2539</td>
<td></td>
<td>1.00 1.05 0.66 0.70 0.99 0.96 0.99 1.00 0.98 1.03 1.00 1.04 0.99 0.95 0.50 0.60 1.02 1.03 1.02 0.98 1.01 0.95 1.10 3.4</td>
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<td></td>
<td>P052</td>
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<tr>
<td>L-1969</td>
<td></td>
<td>0.61 0.50 0.61 0.48 0.70 0.56 0.49 0.54 0.53 0.62 0.58 1.00 1.04 1.02 0.95 1.00 0.95 1.00 0.89 1.00 1.04 1.02 0.95 1.00 0.89</td>
</tr>
<tr>
<td>A-8</td>
<td></td>
<td>0.51 0.50 0.66 0.97 1.00 0.49 0.45 0.49 0.44 0.44 0.97 0.89 1.00 1.04 1.02 0.95 1.00 0.95 1.00 1.04 1.02 0.95 1.00 0.89</td>
</tr>
<tr>
<td>A-20</td>
<td></td>
<td>0.40 0.86 0.98 0.95 0.99 0.51 0.51 0.96 0.96 0.96 0.99 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97 0.97</td>
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<th>Sample #</th>
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<th>exon deleted/duplicated exons, as assessed by qPCR</th>
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<tr>
<td></td>
<td>P099</td>
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<tr>
<td>L-1421</td>
<td></td>
<td>0.99 1.05 0.49 1.00 1.04 5 (het del)</td>
</tr>
<tr>
<td>L-1422</td>
<td></td>
<td>1.04 0.57 0.43 0.81 1.13 5 (het del)</td>
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</table>

Aberrant ratios (in bold) between the exon-specific band volumes and the average value of the corresponding bands of the two mutation-negative controls, for different (a) Parkin, (b) GCH1, (c) SGCE mutation-carrying samples.
The results for the sample T-1 differed between the two different MLPA kits (P051 and P052), where only the mutational status obtained with the P052 kit matched the qPCR results (heterozygous deletion of Parkin exon 3). The screen with the P051 kit indicated an additional heterozygous deletion of Parkin exon 9. T-1 had previously been established to carry a heterozygous dinucleotide deletion in this exon (c.1147-8delAA). An alignment of the exonic sequence and oligonucleotide probes sequences specific for exon 9 of Parkin (from the P051 kit) revealed that the two deleted nucleotides were situated exactly at the 3’ end of the first oligonucleotide, creating a mismatch when annealed to the allele with the mutation (Fig. 2).

In the sample L-324, compound heterozygous Parkin mutations (deletion of exons 3 and 4, and duplications of exons 7 and 9) had been reported. The MLPA analysis with both Parkin probes-containing kits detected a heterozygous duplication of exons 7–12 in addition to the heterozygous deletion of exons 3 and 4. The MLPA of this sample was performed with a newly extracted DNA from a fresh blood sample, and these results were later confirmed by qPCR using the same new sample.

In 49 samples with unknown mutational status, we detected two exonic rearrangements in one sample each (data to be presented elsewhere).

**DISCUSSION**

MLPA not only was able to precisely detect all present changes in our set of samples, but also was, in some instances, able to identify small mutations (c.1147-8delAA in T-1). Therefore, when a decrease in the relative ratio of a band is observed, it is necessary to screen a corresponding exon with another probe or to sequence it to rule out the possibility of a small sequence change.

We did not have the opportunity to investigate samples with SNCA, PINK1, DJ1, LRRK2, or TH exon rearrangements. However, given the high accuracy of the method in detecting Parkin, GCH1, and SGCE exonic deletions/multiplications, we believe that it would be equally successful in detecting those, as well.

MLPA, with its ability to analyze most of the exons of all known genes implicated in PD, DRD, and M-D in only three runs, was easy to perform, fast, and relatively inexpensive. Not taking into account the reduced amount of work, the costs for MLPA consumables are 2 to 9 times lower than those required to obtain the same amount of data with qPCR with hybridization probes. As with all other gene dosage assessment methods, a sufficient amount and high quality of DNA is crucial for a successful analysis. The DNA amount required for MLPA and qPCR is comparable, when considering that each exon of each gene has to be investigated separately and at least in duplicate with qPCR. Performing MLPA on a LI-COR instrument, we obtained the best results (normal ratio values close to 1.0 and aberrant ratios closest to 0.5, 1.5, or 2) with an amount of ~300 ng of high quality DNA for a single reaction.

Despite the many advantages of the MLPA analysis, it is necessary to keep in mind that this method can only detect exonic rearrangements and specific point mutations for which probes are available. Thus, to perform a
comprehensive mutation analysis, qualitative screening methods such as SSCP, dHPLC, or sequencing have to be included.

Employing a new technique for gene dosage testing of movement disorder genes reaches far beyond a simple technical advancement and has two major implications: (1) By improving the availability of comprehensive genetic testing, it supports clinicians in the establishment of a genetically defined diagnosis and minimizes the number of erroneously classified “mutation-negative” patients; (2) By enabling gene dosage testing of several genes simultaneously, it significantly facilitates the mutational analysis of large patient and control populations, which is the prerequisite for meaningful phenotype–genotype correlations.

Many movement disorders, such as PD, are largely genetically heterogeneous and different forms are often indistinguishable on a clinical basis. Furthermore, partial clinical overlap exists between some movement disorders (e.g., PD and DRD). Thus, a combined test for exon rearrangements in all known genes associated with PD and DRD allows for an improved molecular differentiation.

It needs to be stressed, however, that genetic testing for movement disorders should not be recommended lightly in clinical practice, especially in the absence of testing guidelines. Unlike in the case of genes causing, for example, repeat expansion disorders, the major obstacles in interpreting testing results of many PD and dystonia genes are the technical challenges and the cost involved with the molecular analysis of these large genes harboring different mutation types. Therefore, establishing MLPA as a reliable and time- and cost-saving method for fast gene dosage screening will significantly improve molecular testing of various movement disorder genes in both the diagnostic and the research setting.

Acknowledgments: This work was supported by grants from the Deutsche Forschungsgemeinschaft (436 JUG 17/2/05 to AD and CK), the Volkswagen Foundation (Lichtenberg Grant to CK), and the University of Lübeck (MG). AMK is a member of the German Muscular Dystrophy Network (MD-NET 01GM0302) funded by the German Ministry of Education and Research (BMBF, Bonn, Germany).

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