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ORIGINAL ARTICLE

Utility of MLPA in deletion analysis of *GCH1* in dopa-responsive dystonia

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Abstract We applied multiple ligation-dependent probe amplification (MLPA) to patients from three families with characteristic dopa-responsive dystonia (DRD) but no base change in the gene *GCH1*. We found a complete deletion of *GCH1* in affected members of family 1, and partial deletions in affected individuals of family 2 (exons 4–6) and of family 3 (exons 2–6). The findings were confirmed by quantitative real-time PCR. Our investigations demonstrate the utility of MLPA for routine deletion analysis of *GCH1* in DRD patients with no sequence changes in this gene.

Keywords Dopa-responsive dystonia · DRD · *GCH1* deletions · MLPA · qPCR

Introduction

The full-blown clinical picture of dopa-responsive dystonia (DRD, Segawa syndrome) is characterized by generalized dystonia, diurnal fluctuation of symptoms, and a dramatic therapeutic response to L-dopa [1]. However, the clinical spectrum of the disorder is much wider and can range from subtle neurological signs and symptoms (e.g., abnormal writing tests) to orthopedic signs (e.g., pes equinovarus), parkinsonism, and even psychiatric manifestations [2, 3].

DRD is 2.5-fold more common in women than in men [4]. The disorder is inherited as an autosomal dominant trait. Penetrance is reduced and ranges from 30 to 60% in men, depending on whether uncommon and subtle symptoms are recognized as manifestations of the disorder. In women, penetrance is higher and one study suggests almost complete penetrance if patients are carefully investigated for discrete signs and symptoms [5].

In about 50% of cases of DRD, a mutation is detected in the gene *GCH1* that codes for GTP cyclohydrolase 1 (GTPCH1), the rate-limiting enzyme in the synthesis of biopterin [6]. All major types of mutations, including missense, nonsense, splice site mutations and duplications, and deletions, have been reported in patients [2, 7]. Routine mutation analysis, however, focuses on sequencing of *GCH1*, a method that does not detect heterozygous deletions. Until recently, routine deletion analysis was tedious and not feasible to perform in a time- and cost-effective way. It involved either fluorescence in situ hybridization (FISH) and/or quantitative real-time PCR (qPCR) of each one of the six exons of *GCH1*. Therefore, the approximate 50% detection rate of *GCH1* mutations in DRD patients reported in the literature [8] might be an underestimation because deletions are not routinely tested.

To include deletion analysis in routine testing, we applied the recently developed multiplex ligation-dependent primer amplification (MLPA) method to the analysis of deletions of *GCH1* in DRD patients. We studied affected members from

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three DRD families with no sequence changes in *GCH1* and report the discovery of deletions in patients of these families. MLPA proved reliable as demonstrated by confirmation of the results by separate qPCR of the six individual exons.

Materials and methods

DNA extraction and sequencing

DNA was extracted from peripheral blood lymphocytes of patients and controls according to standard procedures. DNA sequencing was done by the dideoxy sequencing method as previously described [8].

Multiple ligation-dependent probe amplification

Exons 1, 2, 3, 5, and 6 of *GCH1* were quantified following the manufacturer's instructions (SALSA P099 GCH1-TH, MRC Holland). 50 to 100 mg DNA were added to the reaction mix provided by the manufacturer. This kit includes primers for exons 1, 2, 3, 5, and 6 of *GCH1* and for the tyrosine hydroxylase gene (*TH*) (see supplier's manual for version 01 of P099). MLPA was performed on a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA). Amplicons were separated on a capillary sequencer (ABI 3100 genetic analyzer, Applied Biosystems, Foster City, CA, USA). Before separation, Genescan-ROX 500 (Applied Biosystems) was added to the samples to facilitate estimation of fragment sizes. Signal data obtained were analyzed using the SeqPilot software (JSI medical systems, Kippenheim, Germany). Relative peak area (RPA) values obtained in probands were compared to those obtained in healthy controls and were expressed as percent ratios. Signal ratios of approximately 50% were considered pathological.

GCH1 deletion analysis by quantitative real-time PCR

For validation of MLPA results, *GCH1* deletion analysis was performed by qPCR. qPCR was performed in the Mx3000p cycler (Stratagene, Amsterdam, The Netherlands) using the manufacturer's Brilliant SYBR Green QPCR Master Mix.

Each reaction (20 µl) contained 10 µl Master Mix, 0.3 µl ROX reference dye (1:500 diluted, included in the Brilliant SYBR Green QPCR Master Mix set), 125 nM forward and reverse primers, and 100 ng DNA. All primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are given in Table 1. The control sample consisted of pooled DNA of 15 independent healthy probands of nonaffected families. *GPR15* was used as reference gene to equalize concentration differences between samples.

Table 1 Primers used for qPCR

Primer	Primer sequence (5′→3′)	Amplicon length (bp)
hGPR15_real1	CAGCAATCAGGAAAGCACAA	87
hGPR15_real2	CAGCCAGGAGACAAGAAAGG	
hGCH1_ex1_real1	CCTACTCGTCCATCCTGAGC	103
hGCH1_ex1_real2	CCCTTGGTGAAGAACTGCAT	
hGCH1_ex2_real1[a]	CCCTCTATCCCTCTTCAGCA	87
hGCH1_ex2_real2[a]	CAGATGATGCCTGAGAGCAC	
hGCH1_ex3_real1[b]	AAGGCACTGTATTTTCACTCAGC	89
hGCH1_ex3_real2	GGCCAAGGACTTGCTTGTTA	
hGCH1_ex4_real1[c]	GCAGTGGTTGGTGGTCTTCT	118
hGCH1_ex4_real2[c]	CCCTGCGATCATGTAAGTCA	
hGCH1_ex5_real1	GAGTCGGGGTAGTGGTTGAA	131
hGCH1_ex5_real2[b]	TCAGTTGTGTGGCATCACCT	
hGCH1_ex6_real1	GCACAATGTTGGGTGTGTTC	141
hGCH1_ex6_real2	CAAGACCGGACAGACAGACA	

[a] Both primers bind in close proximity to the exon (intron 1–2).
[b] One primer of a pair binds in close proximity to the exon.
[c] Both primers bind in close proximity to the exon (intron 3–4).

The real-time PCR cycling conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, and at 60°C for 60 s. After PCR amplification, melt curves were established (55–95°C) to check the accuracy of every PCR amplification. Each sample was run in triplicate. The Ct value, representing the PCR cycle at which the fluorescence emission passes a fixed threshold within the logarithmic phase of the PCR reaction, was measured. *GCH1* exon deletions were determined by the comparative threshold cycle method (ddCt) as previously described [9]: $ddCt=(Ct_{control(reference\ gene)}-Ct_{sample(reference\ gene)})-(Ctcontrol(gene\ of\ interest)-Ct_{sample(gene\ of\ interest)})$. A PCR reaction containing only half of the DNA concentration at a given locus (deletion) requires an additional cycle to reach the same fluorescence intensity as wild-type DNA (no deletion). The ddCt value of a sample with an exon deletion results in 1 under optimal conditions (usual range between 0.6 and 1.4); samples without deletion have ddCt values of 0 (range between −0.4 and 0.4).

Results

Case reports

We investigated three families with apparently dominant inheritance of clinical signs and symptoms consistent with the diagnosis DRD (Fig. 1).

Family 1

In this family three members were affected (Fig. 1a). II/2, presently 66 years old, came to medical attention at the age

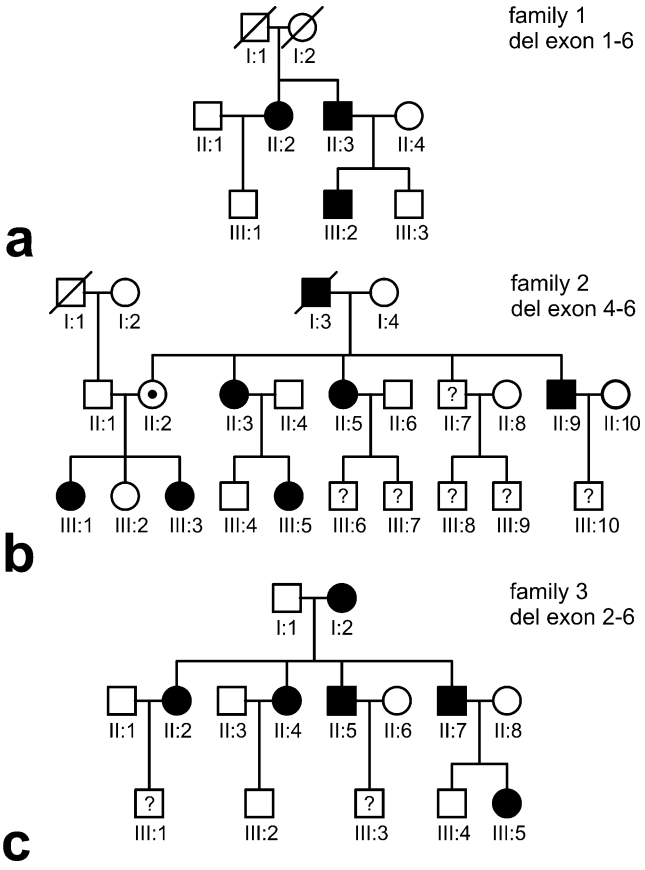

Fig. 1 Pedigrees of the families studied

of 6 because of pes equinovarus of both feet and hyperextension of her back. At age 14, bilateral achillectomy was performed. Her condition worsened and she developed torticollis, involuntary rotation of the left arm, and was at times wheelchair-bound and aphasic. Symptoms deteriorated during the course of the day. After unsuccessful psychotherapy (symptoms were attributed to a psychiatric illness) she was treated with L-dopa starting at age 52 and her symptoms improved dramatically. Presently, her only symptom is an abnormal writing test with involuntary movement of the hand/arm not used for writing. Her brother (II/3), presently 65 years old, developed left pes equinovarus at age 4 and achillectomy was performed early in his life. Presently, his only complaints are "cramping" of both arms and he is not being treated with L-dopa. His 30-year-old son (III/2) had delayed motor development and could not walk without support during infancy. He was treated with L-dopa from age 12 onward and many of his symptoms improved. Supination of the feet and genua valga, however, remained.

Family 2

Three members of this family were severely affected. III/1 developed contraction of all digits of both feet, extreme supination, and cramping of her calves at age 6. Symptoms deteriorated during the day. She is presently 11 years old and her symptoms resolved under treatment with L-dopa. Her only sign is involuntary movement of the contralateral hand when writing. Her sister (III/3) had supination of both feet and pes equinovarus from age 4 onward. Symptoms worsened during the course of the day. She complained of restless sleep. Symptoms improved when L-dopa therapy was started at

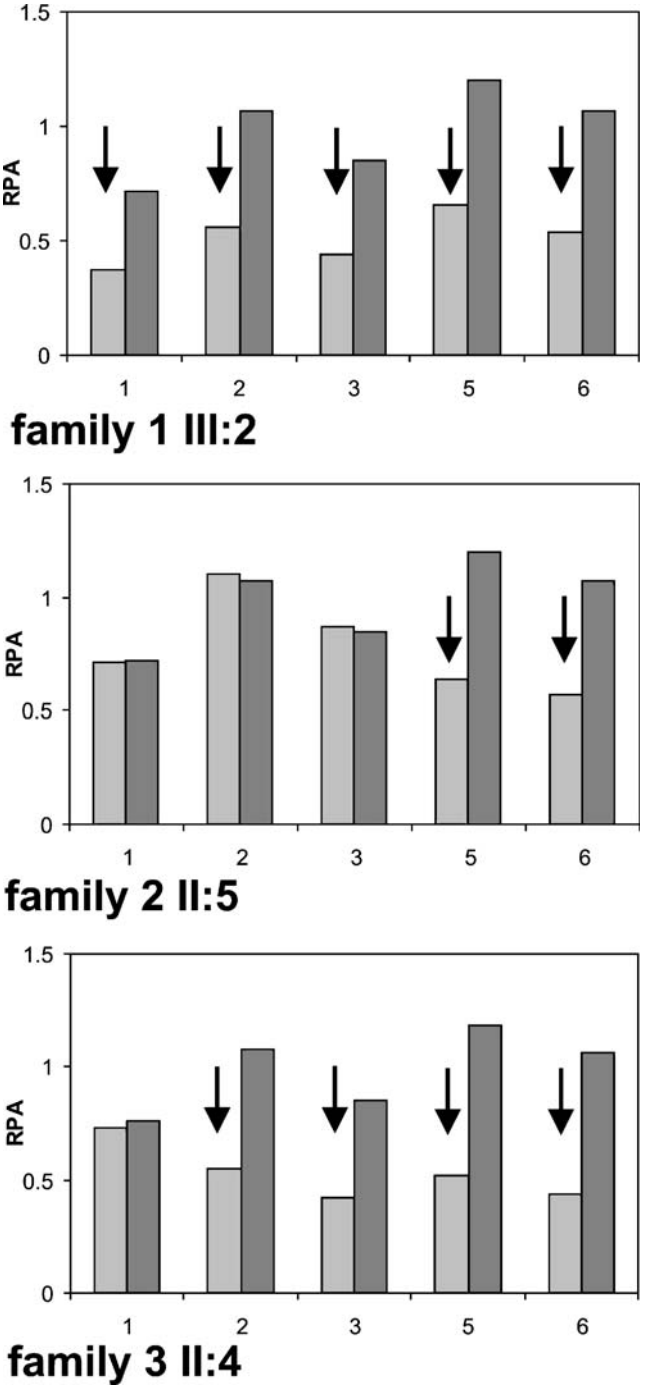

Fig. 2 Results of MLPA analysis for patient III/2 (family 1), II/5 (family 2), and II/4 (family 3). Diagrams show values for peak signals (RPA) of patients (*gray*) compared to signals of controls (*dark gray*). RPA values are given for exons 1, 2, 3, 5, and 6 of GCH1

age 5. Presently, she only displays slight supination of her feet. The sisters' cousin (III/5) developed supination of both feet, kyphosis, and involuntary movements at age 6. Later, torticollis developed and she was surgically treated without success. There was typical circadian variation of symptoms. Under L-dopa since age 11 only an abnormal writing test persists at her present age of 24. Symptoms were also present in the cousin's mother (II/3). She stumbled frequently and showed supination of both feet. She also had an abnormal writing test. Their deceased grandfather (I/3) is reported to have suffered from deformities of the toes and abnormal gait. He had an achillectomy during childhood. Additional mutation carriers of the family (II/2, II/3, II/5, and II/9) had no clinically relevant symptoms. However, they presented with discrete signs of dystonia such as an abnormal writing test.

Family 3

The index patient (II/4) is presently 52 years old. She has suffered from unsteady gait and frequent falls since age 8. Later on she presented with dystonic positioning of her fingers, propensity to fall, reduced positional reflexes, writing cramp, and chronic constipation. At the beginning of her third decade she developed torticollis. At age 34 a stereotactic operation (thalamo-subthalamotomy) was performed without improvement of symptoms. At age 49 an oral phenylalanine loading test was performed. Four hours after administration, a pathologically high phenylalanine/tyrosine ratio of 8.28 was found. Upon administration of L-dopa (4×50 mg/die) all signs and symptoms including constipation have improved dramatically. However, spasticity of the left leg remains and is probably a residual of the stereotactic operation. The patient's sister (II/2) is 54 years old and is reported to have gait abnormalities, cramping of the legs, and malpositioning of hands and legs since age 10. No neurological examination was performed because the patient refuses professional care. In his midthirties, her brother (II/5) only exhibits tremor of the head. The tremor does not interfere with his daily life. Her second brother (II/7, currently 37 years old) displays clumsy gait. His daughter (III/5, presently 6 years old) started to walk at age 2, had a clumsy gait, and suffered frequent falls. At age 4 treatment with L-dopa (4×30 mg/die) was initiated and her symptoms have improved dramatically. No clinical abnormalities were reported in the remaining family members of generation III (see Fig. 1).

Molecular analysis

We sequenced all six exons including intron–exon boundaries of *GCH1* in affected members of the three families and did not detect a sequence change. Because the clinical picture of affecteds was quite characteristic of DRD, we performed deletion analysis using a recently developed MLPA kit (Materials and methods) that allows for detection of deletions/duplications of exons 1, 2, 3, 5, and 6 but not of exon 4. MLPA analysis revealed a heterozygous complete deletion of *GCH1* in patients of family 1, a partial deletion, including exons 5 and 6 in family 2, and another partial deletion including exons 2–6 in family 3.

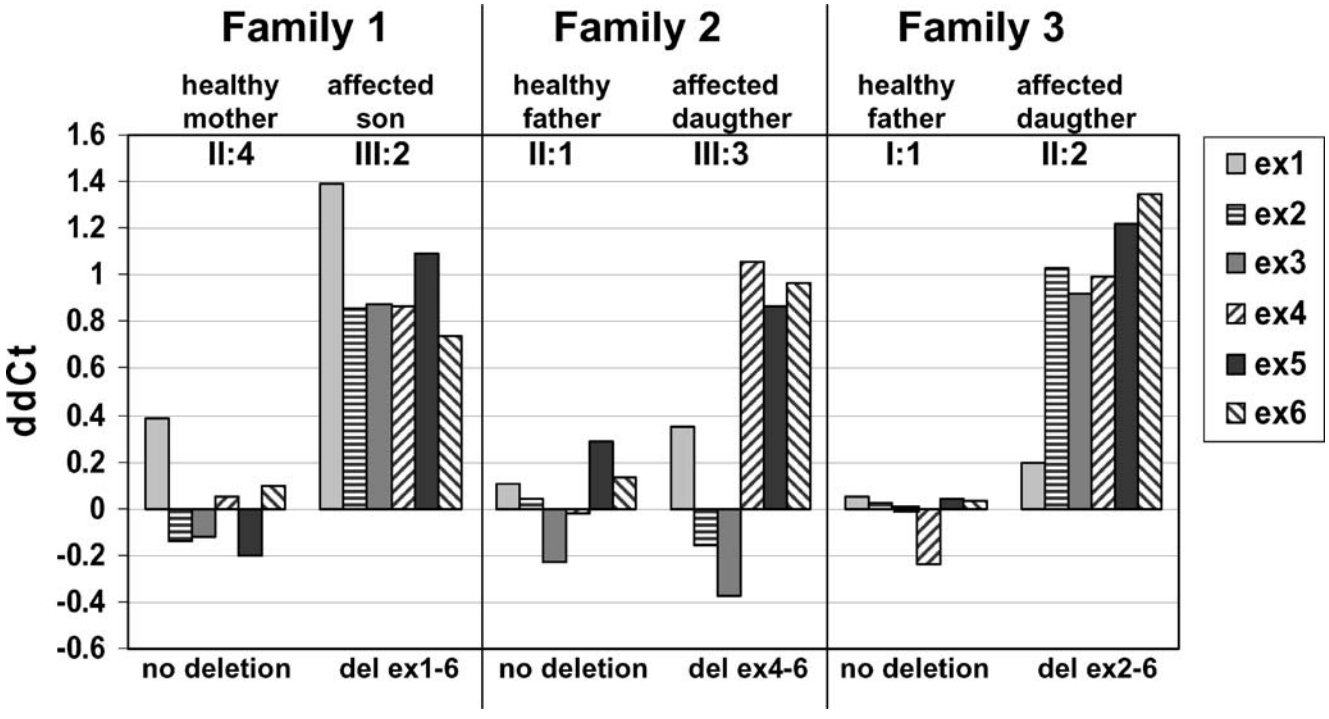

Fig 3 *GCH1* deletion analysis by qPCR in families 1, 2, and 3. ddCT refers to comparative threshold cycle method. For details, see text

Results were confirmed by individual qPCR of all six exons in the index patients of the three families. This analysis also demonstrated that exon 4 is deleted in patients of family 2. The results of MLPA and qPCR are given in Figs. 2 and 3.

Discussion

We have demonstrated that MLPA is suitable for the detection of deletions of *GCH1* in DRD in a routine setting. It is reliable and faster than other tests that involve the separate analysis of individual exons.

The occurrence of complete deletions of *GCH1* in DRD (see also 10–12]) demonstrates that haploinsufficiency of *GCH1* is sufficient to cause DRD. This is not consistent with previous findings that suggest a dominant-negative effect of *GCH1* mutations. Based on the observation of reduction of GTPCH1 activity to significantly less than 50% in patients with heterozygous *GCH1* missense mutations [6], it was thought that mutant and wild-type monomers form dys- or nonfunctional GTPCH1 heterodecamers. This notion was supported in cotransfection experiments using wild-type and mutated *GCH1* cDNA [13]. On the other hand, formation of heterodecamers was not found for at least two mutations (R88W and R184H) of GTPCH1 [14]. To settle the issue of the molecular mechanism of *GCH1* mutations, GTPCH1 activity needs to be determined in patients with heterozygous deletions. Reduction of enzyme activity by approximately 50% would prove that haploinsufficiency is indeed a molecular mechanism in DRD.

The signs and symptoms of patients of the three families are quite characteristic of DRD. There was childhood onset in some with dystonia of the legs and worsening of symptoms during the course of the day. Other mutation carriers were only mildly affected and some did not even consider their symptoms serious enough to seek professional help (e.g., pts. I/2, II/2, II/5, and II/7 of family 3). The patients of the three families further document the importance of simple tests to induce symptoms during clinical examination. Thus, writing can cause dystonic movements in the contralateral hand or leg in otherwise completely normal mutation carriers. Furthermore, the history of some of the patients demonstrates the need of expeditious diagnosis of DRD to avoid unnecessary or harmful medical interventions. The diagnosis DRD should be considered in patients with gait abnormalities, involuntary movements, a positive family history of movement disorders including Parkinson disease and tremor, therapeutic response to L-dopa, and/or circadian fluctuation of symptoms. When some or all of these findings apply to a patient, molecular testing for *GCH1* mutations should be performed. The tests should include sequencing of *GCH1* and—if no sequence change is detected—deletion analysis. The latter can be reliably performed by MLPA. However, the presently available MLPA-*GCH1* kit does not allow for deletion analysis of the small exon 4 (32 bp). Therefore, this exon needs to be separately investigated for a deletion by qPCR, as demonstrated.

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