doi:10.1093/brain/awp084 Brain 2009: 132; 1753–1763 | 1753

BRAIN
A JOURNAL OF NEUROLOGY

Exhaustive analysis of BH4 and dopamine biosynthesis genes in patients with Dopa-responsive dystonia

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Dopa-responsive dystonia is a childhood-onset dystonic disorder, characterized by a dramatic response to low dose of L-Dopa. Dopa-responsive dystonia is mostly caused by autosomal dominant mutations in the *GCH1* gene (GTP cyclohydrolase1) and more rarely by autosomal recessive mutations in the *TH* (tyrosine hydroxylase) or *SPR* (sepiapterin reductase) genes. In addition, mutations in the *PARK2* gene (parkin) which causes autosomal recessive juvenile parkinsonism may present as Dopa-responsive dystonia. In order to evaluate the relative frequency of the mutations in these genes, but also in the genes involved in the

Received November 19, 2008. Revised February 13, 2009. Accepted February 23, 2009. Advance Access publication June 2, 2009

biosynthesis and recycling of BH4, and to evaluate the associated clinical spectrum, we have studied a large series of index patients ($n = 64$) with Dopa-responsive dystonia, in whom dystonia improved by at least 50% after L-Dopa treatment. Fifty seven of these patients were classified as pure Dopa-responsive dystonia and seven as Dopa-responsive dystonia-plus syndromes. All patients were screened for point mutations and large rearrangements in the *GCH1* gene, followed by sequencing of the *TH* and *SPR* genes, then *PTS* (pyruvoyl tetrahydropterin synthase), *PCBD* (pterin-4a-carbinolamine dehydratase), *QDPR* (dihydropteridin reductase) and *PARK2* (parkin) genes. We identified 34 different heterozygous point mutations in 40 patients, and six different large deletions in seven patients in the *GCH1* gene. Except for one patient with mental retardation and a large deletion of 2.3 Mb encompassing 10 genes, all patients had stereotyped clinical features, characterized by pure Dopa-responsive dystonia with onset in the lower limbs and an excellent response to low doses of L-Dopa. Dystonia started in the first decade of life in 40 patients (85%) and before the age of 1 year in one patient (2.2%). Three of the 17 negative *GCH1* patients had mutations in the *TH* gene, two in the *SPR* gene and one in the *PARK2* gene. No mutations in the three genes involved in the biosynthesis and recycling of BH4 were identified. The clinical presentations of patients with mutations in *TH* and *SPR* genes were strikingly more complex, characterized by mental retardation, oculogyric crises and parkinsonism and they were all classified as Dopa-responsive dystonia-plus syndromes. Patient with mutation in the *PARK2* gene had Dopa-responsive dystonia with a good improvement with L-Dopa, similar to Dopa-responsive dystonia secondary to *GCH1* mutations. Although the yield of mutations exceeds 80% in pure Dopa-responsive dystonia and Dopa-responsive dystonia-plus syndromes groups, the genes involved are clearly different: *GCH1* in the former and *TH* and *SPR* in the later.

Keywords: Dopa-responsive dystonia; *GCH1* gene; *SPR* gene; *TH* gene; *PARK2* gene

Abbreviations: CRE = cyclic monophosphate response element; CSF = cerebrospinal fluid; DRD = L-Dopa-responsive dystonia; PCR = polymerase chain reaction; PTS = 6-pyruvoyl tetrahydropterin synthase; QDPR = dihydropteridin reductase; SPR = sepiapterin reductase

Introduction

L-Dopa-responsive dystonia (DRD) is a disorder characterized by childhood or adolescence onset dystonia sometimes associated with mild parkinsonism (Segawa *et al*., 1976). The motor symptoms usually fluctuate during the day and are improved by sleep. The hallmark of the disease is a dramatic and sustained improvement of the dystonia with a low dose of L-Dopa. DRD can also present in adulthood as focal dystonia or parkinsonism. Women are affected 2.5–4 times more frequently than men (Nygaard, 1995).

Most cases of DRD are caused by heterozygous point mutations or, more rarely, large deletions in GTP cyclohydrolase1 (*GCH1*) gene, located on chromosome 14q and encoding the GTP cyclohydrolase 1 enzyme (GTPCH EC 3.5.4.16) (Ichinose *et al*., 1994). GTPCH is involved in the first and rate-limiting step of the *de novo* biosynthesis of tetrahydrobiopterin (BH4) by catalysing the formation of dihydroneopterin triphosphate from GTP (Fig. 1). BH4 is an essential cofactor required for the activity of various enzymes such as the nitric oxide synthases and phenylalanine-, tryptophane- and tyrosine- hydroxylases (Thony *et al*., 2000). The second and last steps of the *de novo* biosynthesis of BH4 are catalysed by the pyruvoyl tetrahydropterin synthase (PTPS EC 4.6.1.10; gene symbol *PTS*) and the sepiapterin reductase (SR EC 1.1.1.153; gene symbol *SPR*), respectively. Furthermore, two additional enzymes, pterin-4a-carbinolamine dehydratase (PCD EC 4.2.1.96; gene symbol *PCBD*) and dihydropteridin reductase (DHPR EC 1.6.99.7; gene symbol *QDPR*), are required for the regeneration of BH4 from intermediates formed during the hydroxylation of aromatic amino acids.

Deficits in other enzymes involved in the BH4 biosynthesis may be responsible for the DRD phenotypes associated with other biochemical hallmarks such as hyperphenylalaninemia. Mutations in *PTS* gene commonly induce hyperphenylalaninemia, but a DRD phenotype has rarely been described (Hanihara *et al*., 1997) since hyperphenylalaninemia is systematically detected through neonatal screening, leading to early substitutive dopaminergic and BH4 treatment. Mutations in the *SPR* gene are responsible for DRD with more complex clinical syndromes (Bonafe *et al*., 2001; Neville *et al*., 2005, Abeling *et al*., 2006), without hyperphenylalaninemia.

Thus, theoretically, all the enzymes involved in the biosynthesis and recycling of BH4 could induce DRD, especially when hyperphenylalaninemia is mild and is not detected at birth. In addition, mutations in two other genes also cause an autosomal recessive form of DRD: the tyrosine hydroxylase gene (TH EC 1.14.16.2; gene symbol *TH*) responsible for typical DRD (Ludecke *et al*., 1995; Furukawa *et al*., 2001; Shiller *et al*., 2004) or L-Dopa-responsive parkinsonism (Ludecke *et al*., 1996; van den Heuvel *et al*., 1998) and the parkin gene (Parkin EC 6.3.2; gene symbol *PARK2*) responsible for juvenile parkinsonism and more rarely for typical DRD (Tassin *et al*., 2000).

However, to date, the relative frequency of the mutations in the genes encoding the enzymes involved in the dopamine and BH4 biosynthesis in DRD patients remains unknown. In order to evaluate the respective contributions of these genes and the associated clinical spectrum, we investigated a cohort of 64 index patients with DRD. All patients were screened for point mutations and large rearrangements in the *GCH1* gene, followed by the sequencing of the *TH*, *SPR, PTS*, *PCBD, QDPR*, as well the *PARK2* genes in patients without mutation in *GCH1*.

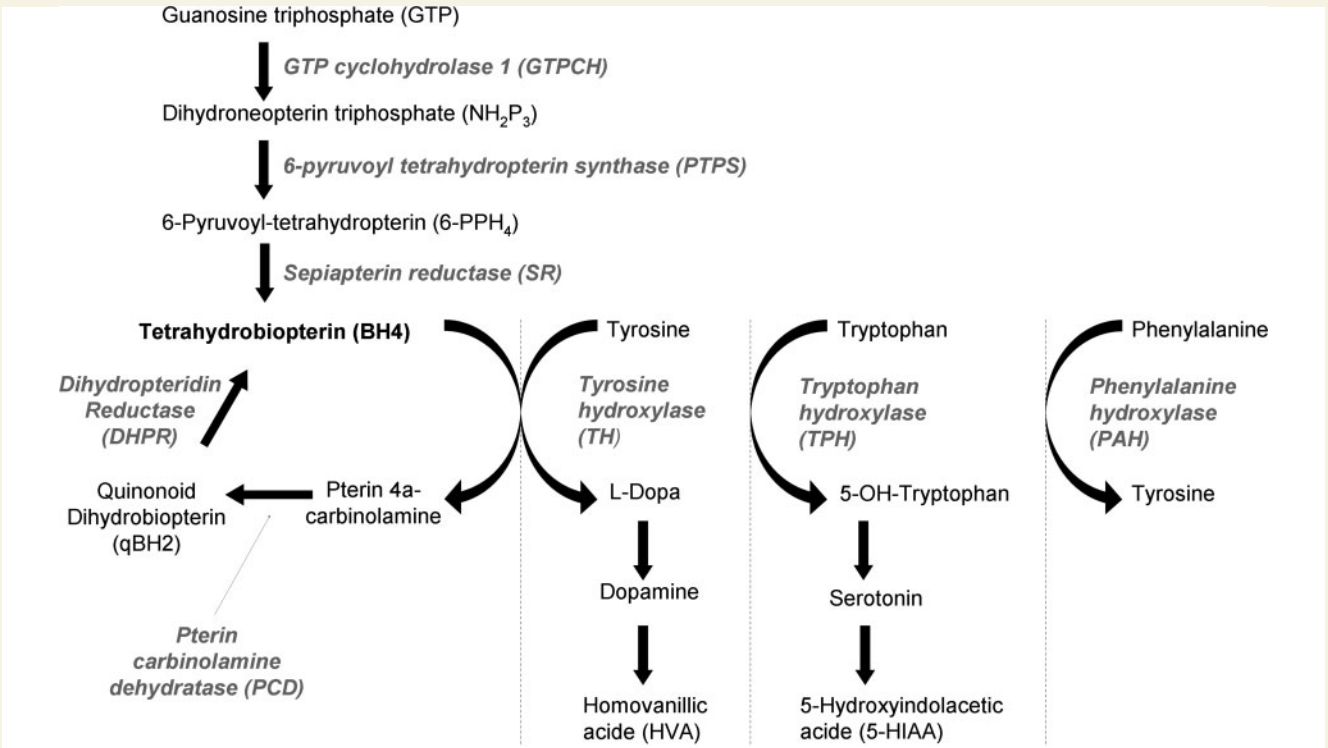

Figure 1 Metabolic pathway of BH4, dopamine, serotonin, and tyrosine.

Patients and Methods

Patients

We selected 64 index patients who were referred to our centre for a molecular diagnosis, and in whom dystonia improved by at least 50% after L-Dopa treatment. Cases with a possible diagnosis of Parkinson's disease either on the basis of clinical evolution or with proven dopaminergic denervation evidenced by 123I-FP-CIT SPECT or fluorodopa PET were excluded from the analysis. Among these patients, 10 had already known point mutations in the *GCH1* gene (SAL37, SAL426, SAL444, SAL445, SAL424, SAL438, SAL452, MON132, CLE150, SAL439) and were described in a previous study (Tassin *et al*., 2000). Their clinical features and mutations are not presented in Table 1, but their data were included in the calculation of the mean age at onset and in the analysis of the relative frequencies of the genes involved in DRD. For patients without mutations in the screened genes, we considered the diagnosis of DRD only if the response to L-Dopa was sustained (i.e. confirmed after at least 1 year of follow-up) and uncomplicated.

Most patients were French; the others originated from Ireland, North-Africa, Mali, Senegal or Guatemala. The medical charts of all the patients were carefully reviewed for clinical data, including age and localization of the dystonia at onset, clinical phenotype of the dystonia at the time of diagnosis and additional neurological features. Pure DRD was defined as isolated dystonia with no other neurological manifestations except mild parkinsonism and a sustained response to low doses of L-Dopa. DRD-plus syndromes were defined as DRD associated with other neurological features (including mental retardation, oculogyric crises, psychiatric manifestations, axial hypotonia and dysautonomia symptoms). Of the 64 patients, 57 had pure DRD and 7 had DRD-plus syndromes. Forty-eight were female and 16 were male. Informed written consent was given by all participating members of the family.

Screening for point mutations in the *GCH1*, *PTS*, *SPR*, *TH*, *PCBD*, *QDPR* and *PARK2* genes

Genomic DNA from peripheral white blood cells was obtained by standard extraction methods (phenol/chloroform). Exons and exon–intron junctions were amplified by the polymerase chain reaction (PCR). Furthermore, the cyclic monophosphate response element (CRE) in the promoter region of the *TH* gene, located between residues −67 and −74 directly upstream of the ATG initiation codon, was amplified by PCR. The amplified fragments were sequenced on an ABI 3730 automated sequencer using the Big Dye 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and the sequencing data were analysed using Sequence Navigator or SeqScape 2.5 software (Applied Biosystems, Foster City, CA).

To predict, *in silico*, the effect of splice site mutations and missense mutations, we used the splicing site score program (http://rulai.cshl.edu/cgi-bin/New_Alt_Exon_Db/score.pl) and the Polyphen program (http://www.bork.embl-heidelberg.de/PolyPhen/), respectively.

Multiplex ligation-dependent probe amplification (MLPA) of *GCH1*, *TH* and *PARK2* genes

Two MLPA were performed. The first one in all patients without a point mutation in *GCH1* using the SALSA MLPA kit P099 GCH1-TH

Table 1 Clinical features of 37 carriers of *GCH1* mutations

Fam No./Sex	Predicted protein alteration	Nucleotide change	Exon	Age of onset (years)	Age at examination	Type of dystonia	Site of onset	Response to L-dopa (%)	Family history
Nonsense mutations									
ITD286/1	p.Glu65X	c.193G>T	1	6	13	Generalized	NK	70	No
ITD451/2	p.Ser81X	**c.242C>A**	1	8	28	Focal	foot	100	No
ITD238/2	p.Trp96X	c.287G>A	1	8	37	Generalized	LL	100	No
ITD709/2	p.Gln188X	**c.562C>T**	5	17	25	Focal	foot	100	No
ITD704/2	p.Arg216X	c.646C>T	6	1.5	11	Generalized	LL	90	No
Missense mutations									
ITD625/2	p.Pro69Leu	**c.206C>T**	1	27	30	Segmental	LL	80	No
ITD699/1	p.Ala99Pro	**c.295G>C**	1	6	30	Segmental	LL	100	No
ITD210/1	p.Phe104Leu	**c.312C>A**	1	4	9	Generalized	LL	95	No
UF298/2	p.Pro147Gln	**c.440C>A**	2	15	19	Focal	foot	100	No
UF237/2	p.Val152Asp	**c.455T>A**	3	Childhood	33	Focal	foot	100	No
UF211/1	p.His153Arg	**c.458A>G**	3	6	20	Multifocal	LL	95	Yes
ITD418/2[a]	p.Ile154Ser	**c.461T>G**	3	5	9	Segmental	LL	100	No
ITD298/2	p.Gln180Pro	**c.539A>C**	4	5	48	Hemi	LL	100	Yes
ITD615/2	p.Val181Ile	**c.541G>A**	4	9	13	Segmental	LL	90	No
ITD530/2	p.His210Arg	**c.629A>G**	6	19	21	Multifocal	foot	100	NK
Splicing mutations									
ITD583/2	Possible exon skipping	**c.344-5T>G**	2	<10	10	Generalized	NK	80	No
ITD722/2	Possible exon skipping	**c.453+1G>A**	2	8	28	Generalized	LL	100	Yes
UF141/1	Possible exon skipping	**c.509+1G>A**	3	8	11	Hemi	Foot	80	No
ITD686/2	Possible exon skipping	**c.509+5G>A**	3	1	38	Generalized	LL	90	No
ITD321/2	Possible exon skipping	**c.626+1G>T**	5	1.5	13	Generalized	LL	80	No
ITD429/1	Exon 5 skipping	c.626+1G>A	5	7	12	Focal	Foot	100	No
ITD537/2	Exon 5 skipping	c.626+1G>A	5	4	33	Segmental	LL	100	No
Small deletions									
ITD588/2[a]	p.Gln48AlafsX16	**c.141_142del**	1	5	10	Hemi	NK	100	No
ITD173/2	p.Lys107ArgfsX8	**c.320_329del**	1	8	28	Segmental	LL	100	Yes
UF190/2	p.Leu179del	**c.536_538del**	4	0.2	3	Segmental	LL	100	Yes
ITD487/2	p.Met211ValfsX38	c.631_632del	6	6	50	Generalized	LL	100	Yes
ITD661/2	p.Met211ValfsX38	c.631_632del	6	12	44	Hemi	NK	80	No
UF251/2	p.Met211ValfsX38	c.631_632del	6	Childhood	22	Hemi	LL	100	Yes
UF291/1	p.Glu236ArgfsX10	**c.706del**	6	6	8	Multifocal	LL	80	Yes
5′-UTR									
ITD627/1		−22C>T 5′-UTR		6	10	Segmental	LL	70	No
Large deletions									
ITD435/1	Absence of transcript	del exon 1	1	11	51	Segmental	LL	100	Yes
SAL434/2	Absence of transcript	del exon 1-6	1–6	8	25	Segmental	LL	100	Yes
ITD612/2[b]	Absence of transcript	del exon 1-6	1–6	1	12	Generalized	LL	100	No
ITD637/2	Absence of transcript ?	del exon 2-6	2–6	5	12	Segmental	LL	80	No
UF222/2	Absence of transcript ?	del exon 2-4	2–3	7	34	Segmental	LL, UL	80	No
ITD677/2	Absence of transcript ?	del exon 4-6	5–6	2	27	Generalized	LL	90	Yes
ITD566/2	Absence of transcript ?	del exon 6	6	6	37	Generalized	LL	100	No

a *de novo* mutation.

b Patient with DRD plus syndromes. New mutations are indicated in bold. The ten *GCH1* mutations of the paper of Tassin *et al*., 2001 are not described here. Large deletions were analysed by MLPA.

Sexe: 1 = male and 2 = female; NK = not known; LL = lower limbs; UL = upper limbs.

(MRC-Holland, Amsterdam, The Netherlands) and the second one in the patients without mutations in the genes involved in metabolic pathways of BH4 and L-Dopa using the SALSA MLPA kit P052B Parkinson.

All of the exons in *GCH1*, except the small exon 4 (32 bp), were quantified. Only 6 of the 14 *TH* exons were quantified. One hundred nanograms of DNA were used in the MLPA protocol, according to the manufacturer's instructions. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems). One microlitre of the PCR products was analysed by capillary electrophoresis on an ABI 3730 automated sequencer and MLPA data were analysed using the ABI Prism Genemapper 4.0 software (Applied Biosystems, Foster City, CA). Relative ratios were calculated for each peak using the formula r = mean (peak area patient/control area patient)/(peak area control individual/control area control).

Quantitative real-time PCR

Exon 4 of *GCH1* was quantified by quantitative real-time PCR (q-PCR) in all patients without point mutations. The primer pairs were designed using Primer Express 1.5 software. The sequence of the forward and reverse primers was TGATTTGTGATTAACTAAAACAATTCTTTC and ACAGATTTTTAAAGCTTACCTTGTAGTCTTC, respectively. Real-time PCR was performed on the ABI PRISM 7500 Sequence detection system (Applied Biosystems), in a total volume of 25 µl, containing 10 ng of genomic DNA, 1× SYBR Green PCR master mix (Applied Biosystems, Foster City, California, USA), and 250 nM of the forward and reverse primers. The standard amplification protocol was used: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To normalize the amount of target DNA, exon 8 of albumin gene was amplified in a separate reaction well under identical thermal cycling conditions. Each reaction was run in triplicate. Copy number values between 0.8 and 1.2 were considered as normal; values $\leqslant 0.6$ evidenced a deletion.

We also quantified exons 2 and 3 of the *SPR* gene by Q-PCR. The sequences of the forward and reverse primers for exon 2 were ACAGTCCTGGCCTCAACAGAA and TGCAGGGCACAGAGGGA, respectively, and the sequences of the forward and reverse primers for exon 3 were GTGAGCTCCCAGGTCATTGG and GCACAGCA CAGACTCCTGACA, respectively.

PCR amplification was performed as above.

Deletion mapping by infinium HD DNA analysis BeadChip

Patient ITD612 was analysed using Illumina's Human CNV 370-Quad BeadChip (Illumina Inc, San Diego, CA, USA). We used 200 ng of patient DNA and followed the protocol as described by the manufacturer (Illumina Inc., San Diego, CA, USA).

Statistical analysis

Age at onset was compared using the Mann–Whitney test. Statistical significance was defined as $P < 0.05$.

Results

GCH1 mutation carriers and their clinical characteristics

Direct sequencing of the *GCH1* gene was performed in 64 unrelated index patients. We identified 38 index patients with heterozygous point mutations in *GCH1*, two index patients with compound heterozygous mutations and seven index patients with heterozygous large deletions. We found 34 different point mutations (6 nonsense, 15 missense, 6 splice site mutations, 6 small deletions and 1 mutation in the 5′ untranslated region). Twenty of them were new mutations (Table 1).

The two patients with compound heterozygous mutations carried a common missense variant (c.68C>T, p.Pro23Leu) located in exon 1. This substitution was previously described as a mutation or a variant in 8 different families (Jarman *et al*., 1997; de la Fuente-Fernandez 1997; Steinberger *et al*., 2000; Scola *et al*., 2007; Zirn *et al*., 2008) and it was found in 1/210 control chromosomes by Jarman *et al*. (1997). We found the p.Pro23Leu variant in 1/174 control chromosomes. The two mutations combined with the p.Pro23Leu variant were a 2-base deletion (c.137_138delGC, p.Ser46SerfsX18) and a missense mutation (c.206 C>T, p.Pro69Leu), both located in exon 1, in patients ITD588 and ITD625, respectively. In patient ITD588, the p.Pro23Leu variant was inherited from the mother and the deletion (c.137_138delGC) occurred *de novo* (segregation analysis of 8 informative polymorphic markers excluded non-paternity). The p.Pro69Leu mutation was found in 1/174 control chromosomes in the same control individual that had the p.Pro23Leu mutation. The parental DNA of the patient and the control were not available, so we could not define whether the mutations were in the *cis*- or in the *trans*- position. The p.Pro23Leu mutation was considered by Polyphen to be benign and the p.Pro69Leu mutation to be deleterious. Moreover, the proline residue at position 23 is not well conserved in other species, in contrast to the proline at position 69. These observations suggest that p.Pro23Leu is a polymorphism and that p.Pro69Leu is a probably causative mutation. All the other missense mutations identified in the present study altered an amino acid that was conserved across mammalian species and were not found on the control chromosomes.

Four splice site mutations (c.453 + 1G>A, c.509 + 1G>A, c.626 + 1G>A and c.626 + 1G>T) affected the invariant bases of the consensus splice donor sequence. The previously reported c.626 + 1G>A splice site mutation (Hirano *et al*., 1998) resulted in the skipping of exon 5 and the introduction of a premature stop codon. The three other splice site mutations were novel; however, since RNA from patients was not available, the transcripts could not be analysed. It is highly probable that these mutations cause exon skipping. We also identified two other splice site variants that did not affect the invariant bases of the consensus splice site sequence, but which were located at position –5 of intron 2 (c.344-5T>G) and at position +5 of intron 3 (c.509 + 5G>A). The mutations reduced the splice site score from 7.2 to 4.4 for the c.344-5T > G mutation and from 11.6 to 8.2 for the c.509 + 5G>A mutation. However, no RNA was available to validate the *in silico* predictions that these variants are splice site mutations.

Except for two in frame deletions (p.Leu179del and p.Arg88_Gln89del), all small deletions caused a frameshift in the coding region that introduced a premature stop codon, theoretically resulting in a truncated protein.

Two of the seven patients with large heterozygous deletions of *GCH1* (SAL434 and ITD612) had complete gene deletions and five had partial deletions (exon 1, exons 2–4, exons 2–6, exons 4–6 or exon 6). Four of the seven patients were sporadic cases, three were familial. In family SAL434, four individuals (two males and two females) had complete deletions. The index case presented a pure DRD, whereas the other patients had only mild equinism. Analysis of microsatellites and SNP in the region flanking the deletion in this family demonstrated that the deletion encompassed only the *GCH1* gene. In patient ITD612 who also had a complete deletion of *GCH1*, a mental retardation was observed in addition to classical signs of DRD. Several male relatives of this patient had a microphthalmia with mental retardation and several females had

low intellectual levels variably associated with strabismus. The size of the deletion was analysed by using Infinium High-Density BeadChips (Human CNV 370-Quad). We identified a 2.3 Mb deletion ranging from rs4901534 to rs1189820. This region contained 10 genes, one of which is the homeobox *OTX2* gene, involved in severe ocular malformations including anophthalmia-microphthalmia and variable developmental delay (Wyatt *et al*., 2008). In family ITD677, three females were affected (the mother and her two daughters). They all had a partial deletion of *GCH1* comprising exons 4–6, with ages at onset ranging from 5 to 8 years. In family ITD435, one female and two males were affected, but DNA was available only for the index patient who had an exon 1 deletion.

In the 47 families with a point mutation or a large deletion in *GCH1*, there were 63 patients and 16 asymptomatic carriers. There were 2.5 times more females than males (45 versus 18) in the patients and 2.2 times more males than females (11 versus 5) in the asymptomatic carriers. Thirty-five percent of the patients had a family history of DRD and 65% were isolated (the one patient for whom the family history was unknown was excluded). Analysis of the DNA of the parents of the five index cases without positive family histories of the disease revealed the existence of two *de novo* mutations (one deletion and one missense mutation).

The clinical characteristics of patients with a mutation or a large deletion in *GCH1* were very stereotyped. The mean age of onset was 7.5 years ± 4.9 (4 months to 27 years) and there was no significant difference between carriers of point mutations compared to carriers of large deletions (7.8 ± 5.1 years versus 5.8 ± 3.2 years, $P = 0.39$). Dystonia started in the first decade of life in 40 patients (85%) and before the age of 1 year in one (2%) (Fig. 2). Onset of the disease after the age of 11 years was observed in six patients (13%), two of which (ITD625 and SAL444-194) manifested the disease after the age of 18 years. Dystonia first appeared in a foot or lower limb in almost all of the patients. The response to L-Dopa was always excellent (70–100% improvement), and the majority of patients experienced diurnal fluctuation of symptoms. Atypical presentations were noted in only three patients who had been diagnosed as having tremor, parkinsonism or spastic paraparesis before confirmation of DRD. Except for mental retardation in patient ITD612, no other neurological features were observed.

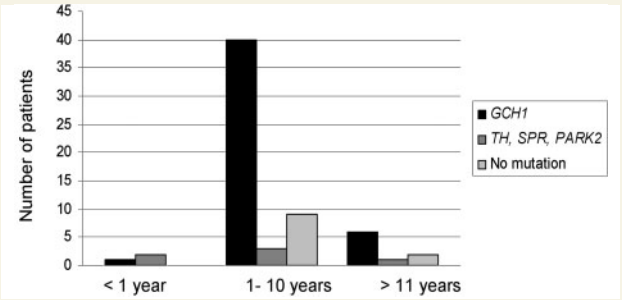

Figure 2 Distribution of ages at onset in patients with *GCH1* (black bars) or *TH/SPR* mutations (dark grey bars), or in patients without mutations (light grey bars).

Mutations in *TH* and *SPR* genes

We analysed *TH* in 14 of the 17 index patients without mutations in *GCH1* and onset before the age of 16 years along with *SPR* in all of the patients. Five different mutations, four of which were new, were found in *TH* in three index patients. Two previously described mutations in *SPR* were found in two index patients (Table 2).

Patients with mutations in the *TH* gene

Patient ITD600, a girl from Guatemala, was examined at the age of 2 years. She had early motor delay with reduced proximal tone that contrasted with dystonia and stiffness in the limbs. These motor symptoms were characterized by marked diurnal fluctuations. However, she had no cognitive dysfunction. Dystonia dramatically improved (by nearly 80%) with low doses of L-Dopa. Analysis of the cerebrospinal fluid (CSF) revealed low levels of homovanillic acid (HVA) and normal levels of 5-hydroxyindole acetic acid (5-HIAA). Detailed clinical and biochemical descriptions of this patient, consistent with a TH deficiency, was described by Doummar *et al*. (2009). This patient had two heterozygous missense mutations c.1125C>G and c.1399A>G, responsible for changes in two conserved amino acids, phenylalanine to leucine at codon 375 (p.Phe375Leu) in exon 10 and serine to glycine at codon 467 (p.Ser467Gly) in exon 13, respectively. Parental DNA was not available because the patient had been adopted. Since the mutations are located in the catalytic domain of the TH protein, they are expected to alter its enzymatic activity.

Patient ITD736, a female, was examined at the age of 24 years. Her disease started during early childhood with progressive motor delay including axial hypotonia and segmental dystonia of the lower limbs with bilateral equinism. When examined, she had generalized dystonia with bulbar involvement, moderate parkinsonism, bilateral rest tremor, mild cognitive impairment and depression. She improved by 80% with low doses of L-Dopa. No biochemical data on pterin metabolism were available. Her brother had similar clinical signs, but the *TH* gene could not be sequenced for lack of DNA. Patient ITD736 carried two heterozygous missense mutations, c.956G>C and c.1240G>A changing two conserved amino acids: arginine to proline at codon 319 (p.Arg319Pro) in exon 9 and glycine to argine at codon 414 (p.Gly414Arg) in exon 12, respectively. The p.Gly414Arg mutation has already been described (Giovanniello *et al*., 2007). The p.Arg319Pro and p.Gly414Arg mutations were not found on 128 control chromosomes and both are located in the catalytic domain of the protein.

Patient ITD797, a girl, was examined at the age of 6 years. He parents were consanguineous. Pregnancy, delivery and the neonatal period were normal. Motor delay was noticed by her mother at the age of 5 months. Her head control was poor and she could not sit. Axial and proximal tone was low but dystonia and spastic stiffness were observed in the extremities limbs. She also had parkinsonism, including episodes of rest tremor, together with multifocal myoclonus. Her deep tendon reflexes were very brisk. Her motor symptoms fluctuated during the day and were improved by rest. In addition, she had supranuclear gaze palsy and mild mental retardation. A few weeks after the initiation of L-dopa treatment, her motor status improved dramatically, and she could

Table 2 Clinical features of patients with mutations in the *TH*, *SPR* or *PARK2* genes

Fam No./Sex	Predicted protein alteration	Nucleotide change	Exon	Age of onset (years)	Age at examination	Type of dystonia	Site of onset	Additional features	Response to L-dopa (%)	Family history
Mutations in *TH*										
ITD600/2[a]	p.Phe375Leu p.Ser467Gly	**c.1125C>G** **c.1399A>G**	10 13	0.5	3	Segmental	LL	Parkinsonism Hypotonia	80	NK
ITD736/2[a]	p.Arg319Pro p.Gly414Arg	**c.956G>C** c.1240G>A	9 12	Childhood	24	Generalized	LL	Parkinsonism Depression	80	Yes
ITD797/2[a]	p.Pro301Ala (homozygous)	**c.901C>G**	8	0.5	7	Segmental	LL	Parkinsonism Mental retardation Hypotonia	90	No
Mutations in *SPR*										
ITD498/2[a]	Possible exon skipping (homozygous)	c.596–2A>G	3	6	13	Generalized	Trunk	Mental retardation Oculogyric crises	90	Yes
ITD613/1[a]	p.Arg150Gly (homozygous)	c.448A>G	2	2.5	23	Generalized	LL	Parkinsonism Oculogyric crises Hypersomolence Hyperphagia	80	No
Mutations in *PARK2*										
ITD729/2	p.Cys449Tyr (homozygous)	**c.1346G>A**	12	16	23	Generalized	UL	Postural and Action tremor of UL	90	No

a Patient with DRD plus syndromes. New mutations are indicated in bold.
1 = male and 2 = female; NK = not known; LL = lower limbs; UL = upper limbs.

control her head and sit normally. Reduced levels of HVA and HVA/5HIAA ratio were found in her CSF. She had a homozygous missense mutation (c.901C>G) that changed a conserved proline to alanine at codon 301 (p.Pro301Ala) in exon 8. This mutation was not found on the 184 control chromosomes and both parents were heterozygous for the mutation.

Patients with mutations in *SPR* gene

Patient ITD498, a girl, was examined at 13 years of age. She was investigated for generalized dystonia with diurnal fluctuations and improved 90% on 50 mg of L-Dopa a day. Her dystonia started in the trunk at the age of 6 years and progressively spread and worsened. Since the onset of her disease, she had episodes, in which her eyes rolled-up, which could last several minutes. Mental retardation was also noted. No biochemical data on pterin metabolism were available. The patient had an affected sister who had the same clinical phenotype since the age of 8 years. The two sisters had homozygous c.596-2A>G mutation in the splice acceptor site of intron 2, and their parents both carried the same mutation in the heterozygous state. This mutation has been described previously (Farrugia *et al*., 2007) and probably results in the absence of the protein.

Patient ITD613, a male, was examined at age 23 years. His disease started during the first year of life with oculogyric crises. He progressively developed generalized dystonia that was first noted in the lower limbs and progressively spread to the trunk, neck and upper limbs. He also had mild parkinsonism without tremor. His symptoms were characterized by marked diurnal fluctuation. Dystonia was significantly improved by L-Dopa, but some oculogyric crises, brought on by exercise, still occurred despite the treatment. The patient also complained of hypersomnolence and hyperphagia. Analysis of CSF revealed very low levels of HVA and 5-HIAA and elevated levels of neopterin and biopterin. This patient had a homozygous missense mutation: c.448A>G in exon 2, changing a conserved arginine to glycine at codon 150 (p.Arg150Gly). The mutation has already been described and results in an inactive protein (Bonafé *et al*., 2001).

Mutation in the *PARK2* gene

In the remaining 12 DRD patients with no mutations in *GCH1*, *TH* or *SPR* genes, the *PTS*, *PCBD* and *QDPR* genes were sequenced. No mutations were detected. Following these results, we analysed by sequencing and MLPA the *PARK2* gene in patients in whom the pattern of transmission was compatible with autosomal recessive inheritance. We found a new homozygous missense mutation c.1346G>A in exon 12, changing a conserved cysteine to tyrosine at codon 449 (p.Cys449Tyr) in patient ITD729. This mutation was not found in 340 control chromosomes and both parents were heterozygous for the mutation. The patient was a female examined at 23 years. She had a generalized dystonia which was improved by 150 mg of L-dopa and a postural and action tremor of the upper limbs. Dystonia started in the upper limbs at 16 years. At seven years follow-up, the L-Dopa was still efficient and no dyskinesia were observed.

Patients without mutations in any of the genes tested

Eleven DRD patients were negatives for all of the genes analysed. Five were sporadic cases, three were familial. The family histories

Table 3 Clinical features of 11 DRD patients without mutations in the genes involved in metabolic pathways of BH4 and L-Dopa and in the *PARK2* gene

Fam No./Sex	Age of onset (year or month)	Age at examination (years)	Duration of FU with L-Dopa	Type of dystonia	Location of first dystonia	Additional features	Response to L-dopa	Family history
ITD249/1	3 month	25	16	Generalized	Neck		90	Yes
ITD328/1	27	30	4	Segmental	UL		50	No
ITD435/2	1	2	2	Segmental	LL		100	Yes
ITD458/2	0.5	41	1	Focal	Neck	Postural tremor of neck and UL	70	No
ITD478/2	6	27	3	Generalized	NK	Limbs spasticity	100	No
ITD592/1	1	18	2	Generalized	NK		100	NK
ITD623/2	8	9	3	Focal	Foot		100	No
ITD670/2	17	30	4	Focal	Hand		100	Yes
ITD682/2	12	31	14	Generalized	LL		100	NK
ITD703/1[a]	5	6	1	Hemi dystonia	NK	Slight mental retardation	90	NK
UF221/2	19	20	1	Multifocal	LL		90	No

a All patients had pure DRD except one patient with DRD-plus syndromes.
1 = male and 2 = female; NK = not known; LL = lower limbs; UL = upper limbs; FU = follow-up.

of the other three patients are unknown. The mean age of the patients at onset was 8.8 ± 8.9 years (3 months to 27 years). Their clinical characteristics are summarized in Table 3. One patient, ITD703, was classified as DRD-plus syndromes because of slight mental retardation. At the latest follow-up evaluation, the improvement under dopaminergic treatment was sustained and uncomplicated for all these patients. No dyskinesia was observed.

Discussion

We looked for mutations in all of the genes involved in the biosynthesis of BH4 and in *TH* gene involved in the synthesis of L-Dopa and in the *PARK2* gene in 64 index patients with DRD, whose motor symptoms improved by at least 50% after L-Dopa treatment (summary, Fig. 3). We found 40 patients (62.5%) with point mutations and seven patients (11%) with large deletions of *GCH1*. Among these 47 *GCH1* positive patients, 76.6% were female. These results were consistent with other studies of DRD patients: a frequency of *GCH1* point mutations of 50–60% and a frequency of *GCH1* large deletions of 8% (Hagenah *et al*., 2005; Zirn *et al*., 2008) with females affected 2.5–4 times more frequently than males (Nygaard, 1995). Fifteen percent of the 47 *GCH1*-positive patients had large deletions, suggesting that MLPA or Q-PCR should be used routinely to search for rearrangements in *GCH1* gene in DRD patients in whom mutations had not been found by sequence analysis. Thus, the redefining of DYT 14 as DYT5 in the paper by Wider *et al*. (2008) has been made possible by using a dosage method.

Thirty-four different *GCH1* point mutations were identified. They are distributed in all of the exons, and all types of mutations were found: missense mutations (37.5%), nonsense mutations (17.5%), splice site mutations (20%), small deletions (20%) and changes in the 5′untranslated region (5%). Three mutations (p.Arg216X, c.626 + 1G>T and 5′UTR-22C>T) and one deletion (c.631_632delAT) were found in more than one subject. For the p.Arg216X nonsense mutation, a common haplotype for four microsatellite markers around the *GCH1* region was previously observed (Tassin *et al*., 2000). Analysis of eight CA repeat markers in the *GCH1* region did not reveal a common haplotype among individuals with the other recurrent mutations, suggesting that they did not originate from a common founder.

Patients with mutations in *GCH1* gene had stereotyped clinical features, characterized by pure dystonia with onset in the lower limbs and an excellent response to low doses of L-Dopa. Only one patient was classified as having DRD-plus syndromes because of mental retardation, probably caused by the 2.3 Mb deletion. *GCH1* mutations accounted for four fifths of the cases with pure DRD and only one case with DRD-plus syndromes. Dystonia generally started in the first decade of life, and in one patient before the first year of age (Fig. 2). However, delayed onset is possible; 6/47 patients first manifested the disease between the age of 11 and 27 years. Five of the six patients had mild dystonia (focal or segmental) and one a hemidystonia. No difference in the clinical presentation or age at onset was observed in carriers of point mutations or large deletions in *GCH1* gene or in patients with different types of point mutations. However, it is interesting to note that three of the four patients with onset after the age of 15 had missense mutations. Some atypical and misleading presentations were found, however, including focal dystonia or pseudo-spastic paraplegia in ITD704. The diagnosis of DRD should therefore be considered in such cases and the efficacy of L-Dopa tested.

Three of the 17 index patients without point mutations or large deletions in *GCH1* gene, in our cohort of 64 DRD patients, had mutations in the *TH* gene and two had mutations in the *SPR* gene, corresponding to frequencies of 4.7% and 3%, respectively. The clinical presentations of these five patients were strikingly more complex and were classified as DRD-plus syndromes. The frequencies of *TH* and *SPR* mutations in these patients with DRD-plus syndromes were 42.8% (3/7) and 28.6% (2/7), respectively.

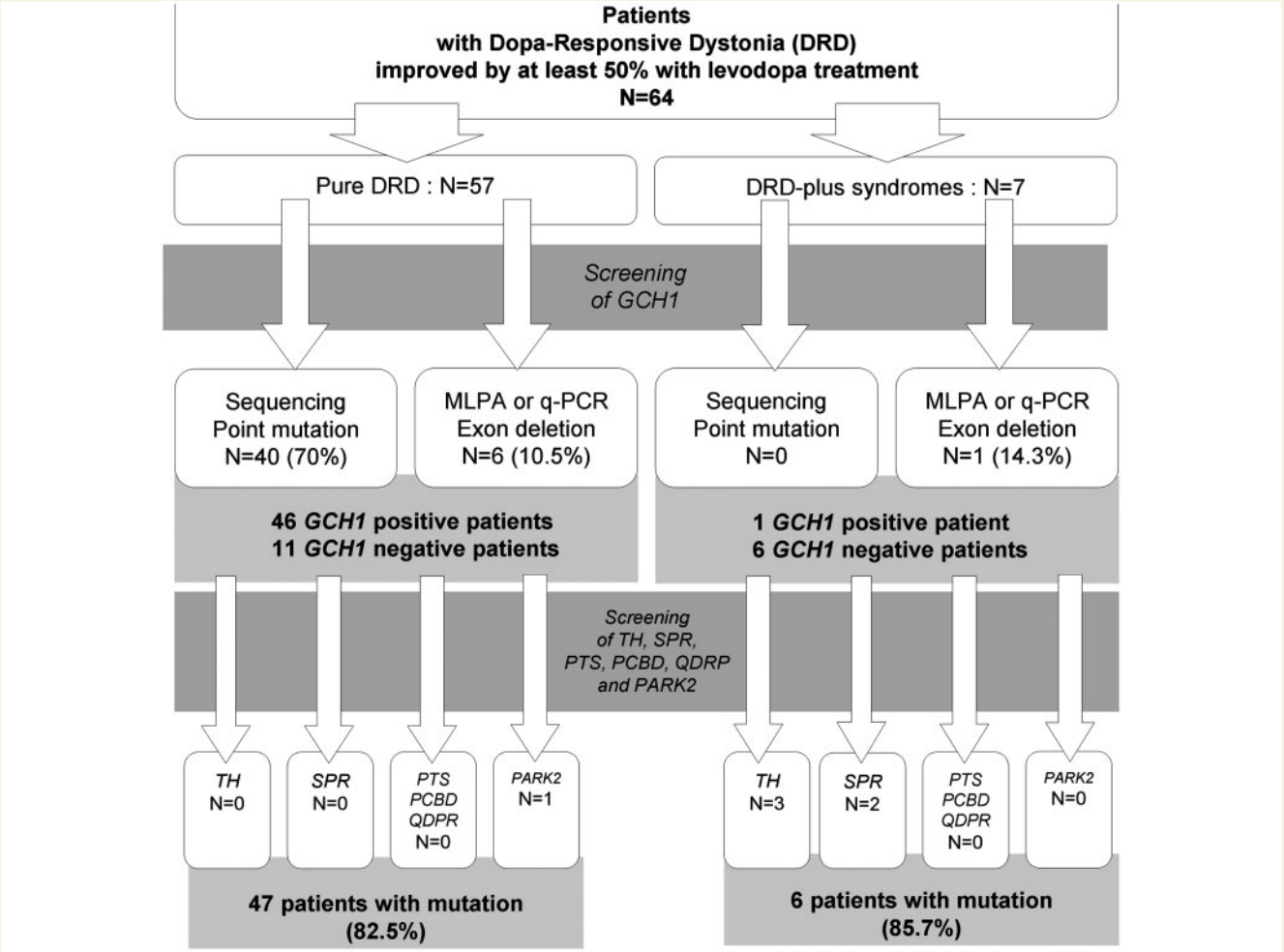

Figure 3 Molecular analyses of six genes involved in the metabolic pathways of BH4 and dopamine synthesis and of the *PARK2* gene in 64 index patients with pure DRD or DRD-plus. GCH1 = GTP cyclohydrolase 1; PARK2 = parkin; PCBD = pterin carbinolamine dehydratase; PTS = 6-pyruvoyl tetrahydropterin synthase; QDPR = dihydropteridin reductase; SPR = sepiapterin reductase; TH = tyrosine hydroxylase.

In patients with *TH* mutations, the disease started early, often in the first year of life. To date, 29 patients with *TH* mutations have been reported in the literature. The clinical presentations of patients with TH deficiency ranged from typical DRD (Ludecke *et al*., 1995; Furukawa et al, 2001; Shiller *et al*., 2004) to L-Dopa-responsive infantile parkinsonism (Ludecke *et al*., 1996, Swaans *et al*., 2000) or severe progressive encephalopathy (Hoffmann *et al*., 2004, Ribasés *et al*., 2007). All of our patients, one of whom was mentally retarded, had infantile parkinsonism with a rather good response to L-Dopa therapy which was limited by the occurrence of dyskinesia. Biochemical analyses of the CSF in two patients revealed decreased HVA levels. In the majority of the patients with mutations in the *SPR* gene described in the literature (Bonafe *et al*., 2001; Neville *et al*., 2005, Abeling *et al*., 2006, Friedman *et al*., 2006), the L-Dopa-responsive movement disorder was associated with other symptoms, such as ataxia, oculogyric crises, dysautonomia symptoms as hypersalivation, microcephaly or growth retardation. Two of our patients had oculogyric crises. In addition, mental retardation was observed in patient ITD498, and patient ITD613 had behavioural features, including sleep disorders or hyperphagia, that might be related to deficits in other neurotransmitters such as serotonin. Analysis of the *TH* and *SPR* genes in DRD patients is not yet part of routine screening. Our results suggest that it should be performed in selected patients with dystonia associated with others symptoms, such as infantile parkinsonism, mental retardation or oculogyric crises but also in negative *GCH1* patients with typical DRD. Indeed, mutations in *TH* or *SPR* genes can both result in a clinical presentation of typical DRD (Ludecke *et al*., 1995; Furukawa *et al*., 2001; Steinberger *et al*., 2004) even if this form is less frequently observed than a complex one.

To investigate all of the enzymes of the BH4 biosynthesis pathway, we sequenced the *PTS*, *PCBD* and *QDPR* genes in the 12 remaining DRD patients without mutations in *GCH1*, *SPR* or *TH* genes. Despite an observation of dystonia in one patient with a mutation in the *PTS* gene (Hanihara *et al*., 1997) we found no mutations in this gene in our DRD patients. This is not surprising, since deficits in these enzymes usually result in

hyperphenylalaninemia, which is detected in newborn children and treated by early administration of L-Dopa and BH4 which prevents the development of dystonia. The identification of a *PARK2* homozygous mutation in one patient of our cohort confirm that defects in this gene can cause DRD similar to DRD secondary to *GCH1* mutations. In Parkinson's disease (either genetic or not), the DRD phenotype seems to be related with a juvenile onset (Lucking *et al*., 2000). In such patients, only studies using 123I-FP-CIT SPECT or [18F]dopa PET scans would allow to rule out dopaminergic cell loss and point to a BH4 or dopamine biosynthesis defect.

Of the 11 patients without mutations, 10 had pure DRD with a mean age at onset of 9.1 years ± 9.3 (range 3 months to 27 years), slightly higher than that of patients with *GCH1* mutations (7.5 years ± 4.8, range 4 months to 27 years). The inability to detect *GCH1* mutations in apparently typical phenotypes was also reported in the papers of Hagenah *et al*. (2005) and Zirn *et al*. (2008) and is still unexplained. Several hypotheses could account for this observation, such as undetected mutations in *GCH1* promotor or regulatory regions leading to a decreased expression of the gene. For these patients the hypothesis of juvenile Parkinson's disease not caused by *PARK2* may also be considered.

The value of measuring pterins and neurotransmitters levels in the CSF was not systematically assessed in this study focused on genetic aspects of DRD. However, these dosages help to guide molecular analyses of *TH* and *SPR* genes in DRD-plus syndromes. Finally, CSF study could be crucial for DRD patients without mutations to formally discriminate between those with neurotransmitters or pterins deficiencies.

In conclusion, patients with isolated DRD and with a good and sustained response to low doses of L-Dopa should be tested, firstly for point mutations, then for large deletions in *GCH1*. Molecular analyses of other genes, including *TH* and *SPR*, should be performed in patients without *GCH1* mutation and in those with DRD-plus syndromes, particularly when onset occurs during the first year of life and before the age of 10. Ideally, the genetic testing should be guided by biochemical analysis of the CSF. Our study also suggests that molecular analyses of others genes involved in hyperphenylalaninemia are likely to be negative. DRD presentation of juvenile parkinsonism should be carefully ruled out by testing patients for *PARK2* mutations and by performing 123I-FP-CIT SPECT or [18F]dopa PET scans. Interestingly, the yield of genetic analyses exceeds 4/5 in patients with pure DRD or DRD-plus syndromes, but the genes involved are clearly different in the two groups: *GCH1* in pure DRD and *TH* or *SPR* in DRD-plus syndromes.

Acknowledgements

We are grateful for the contribution of the patients and their relatives. We wish to thank the clinicians who referred patients to us (Drs Yves Agid, Richard Baronnet, Patrick Berquin, Eric Bieth, Thierry Billette de Villemeur, Emmanuel Broussole, Pierre Burbaud, Patrick Calvas, Jean-Paul Carrière, Christian Confavreux, Pierre Crost, Karin Dahan, Valérie Drouin Garraud, Franck Durif, Bernard Echenne, Laurence Faivre, Annaik Feve, Pierre Genton, Philippe Goas, Lucie Guyant-Marechal, Didier Hannequin, Bertrand Isidor, Pierre Jedynak, Philippe Jonveaux, Alain Lagueny, Caroline Moreau, Jacques Motte, Sylvie Odent, Frédéric Sedel, Marion Simonetta-Moreau, Clémence Simonin, Mohnish Suri, Van Gaever, François Viallet, Jean-Sébastien Vidal, Catherine Vincent-Delorme, Isabelle Vuillaume and Sandra Vukusic). We thank Dr Merle Ruberg for her helpful suggestions on the article (INSERM U679), Ms Lydia Guennec, Isabelle Lagroua and Christelle Dussert (DNA and cell bank of IFR70) for their technical assistance.

Funding

INSERM, Réseau National des Dystonies and GIS-Maladies Rares.

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