

## A splice mutation in the GTP cyclohydrolase I gene causes dopa-responsive dystonia by exon skipping

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**Summary:** Four different mutations in the GTP cyclohydrolase I gene were found (P199L, M211V, IVS5+1G>A, G203R) in 6 out of 33 families with dopa-responsive dystonia. A splice mutation (IVS5+1G>A) located at the border of exon 5 to intron 5 was found in one of these families. Three members of the family carry the IVS5+1G>A mutation on one allele, inherited from the father to the daughter and son. Examination of the mRNA showed an exon 5 skipping that results in a reduction of enzyme activity in cultured fibroblasts to 4–17% compared to controls. The father and daughter never had clinical symptoms of dopa-responsive dystonia. The son was symptomatic at the age of 3 years and was treated successfully with L-dopa/carbidopa. After 20 years this therapy was terminated and for the next 6 years he was free of symptoms. With increased motoric activity, symptoms reappeared and the therapy was reintroduced.

Dopa-responsive dystonia (DRD) is a heterogeneous disorder with autosomal dominant or autosomal recessive inheritance. The main finding is a reduced level of the dopamine metabolite homovanillic acid (HVA) in the cerebral spinal fluid. Dopamine is produced from tyrosine, via L-dopa, by tyrosine-3-hydroxylase (TH; EC 1.14.16.2) (Nagatsu et al 1964). Defects in this enzyme are inherited autosomal recessively (Bräutigam et al 1998; Görke and Bartholomé 1990; Knappskog et al 1995; Lüdecke and Bartholomé 1995; Lüdecke et al 1995). For full activity, TH requires tetrahydrobiopterin as a natural cofactor. GTP-cyclohydrolase I (GTPCH; EC 3.5.4.16) catalyses the first step in the synthesis of tetrahydrobiopterin (Thöny et al 2000). Mutations of this enzyme were found to cause DRD (Bandmann et al 1996; Furukawa et al 1996; Hirano et al 1995; Ichinose et al 1994; Thöny and Blau 1997). The symptoms are dystonic movements and were seen in early childhood. In our

families the symptoms had diurnal variations, being less pronounced in the morning and worsening during the day. Inversed diurnal fluctuation could not be detected (Bandmann et al 1998). The symptoms disappear after low doses of L-dopa/carbidopa (Nygaard et al 1991; Ozelius and Breakfield 1994).

## MATERIALS AND METHODS

*Case report:* Thirty-three families were examined in which at least one member suffered from DRD. One family was examined in more detail. The son exhibited symptoms at the age of 3 years, and was successfully treated with Madopar for 20 years. Then treatment was stopped for 6 years and was started again because DRD symptoms appeared during a period of increased motoric activity. The father and the daughter, who carry the same mutation, never had any symptoms.

*PCR amplification and sequence analysis of genomic DNA:* All exons and the exon-intron boundaries of the GTPCH gene were examined. The primers used have been published by Ichinose et al (1994).

The 100  $\mu$ l PCR reaction tube contained 1  $\mu$ g genomic DNA, 40 pmol of each primer (for exon 2 and 4 this amount was doubled), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% gelatin, 200  $\mu$ mol/L dNTP, 2.5 U Taq polymerase and different concentrations of MgCl<sub>2</sub> (1.5–2.5 mmol/L). The amplification required 37 reaction cycles with three temperature steps: denaturation, 50 s at 95°C; annealing, 45 s at 58–62°C; extension, 3 min 45 s at 73°C; followed by an extension of 13 min 45 s at 73°C. After amplification the DNA fragments were sequenced using the dideoxy method chain-terminating technique (Sanger et al 1977; Wong et al 1987).

*Isolation and transcription from mRNA into cDNA:* mRNA was isolated from peripheral mononuclear cells. To transcribe mRNA into cDNA the following mixture was used: 2.56  $\mu$ g total RNA, 5  $\mu$ l bulk first-strand cDNA reaction, 1  $\mu$ l (200 mmol/L) dTT solution, 1  $\mu$ l (0.2  $\mu$ g) pd (N<sub>6</sub>) primer. The total volume was 15  $\mu$ l and incubation time was 1 h at 37°C.

*cDNA polymerase chain reaction:* Two primers were used: C<sub>5</sub>, 5'-AACCAATAGAAGGATTGTTC-3'; and Oligo 1000, 3'-ACGATTATCACATTCATGCA-5'. These primers cover the borderline from exon 5 to intron 5. The PCR reaction mixture had a total volume of 50  $\mu$ l containing 8–15  $\mu$ l cDNA and 20 pmol primer C<sub>5</sub> and Oligo 1000, 5  $\mu$ l 10 $\times$  PCR buffer, 15 mmol/L MgCl<sub>2</sub>, 2  $\mu$ l dNTPs (50  $\mu$ mol/L A, C, G and T) and 2.5 U Taq polymerase. The amplification was done in 30 cycles at three different temperatures: 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 73°C.

*GTPCH activity and pterin levels:* Pterins (total neopterin and biopterin) and GTPCH activity were measured in cytokine-stimulated fibroblasts (Bonafé et al 2001).

**Agarose gel electrophoresis:** To visualize the PCR product, 5  $\mu$ l of the reaction mixture and 5  $\mu$ l of gel loading buffer were applied on a 1% agarose gel (1% agarose, 1% NuSieve agarose, 1 $\times$  TBE buffer). In parallel a molecular weight marker (9–587 bp, Boehringer Mannheim) was added.

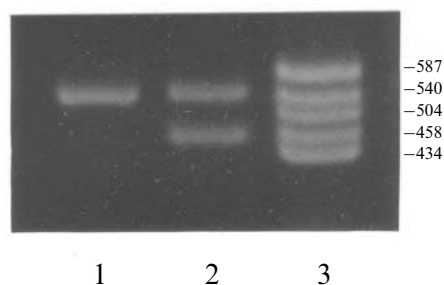
## RESULTS

We examined 33 families in which at least one member suffered from DRD and found four different mutations in six families in the GTP cyclohydrolase I gene (P199L, M211V, IVS5+1G>A, G203R). The G203R mutation was found in three families. All mutations were inherited autosomal dominantly. In one family the mutation G203R occurred spontaneously and the fatherhood was proved by a genetic fingerprint. In the six families with a mutation in the GTPCH I gene, there were 12 patients (6 women, 6 men) and 8 asymptomatic carriers (2 women, 6 men).

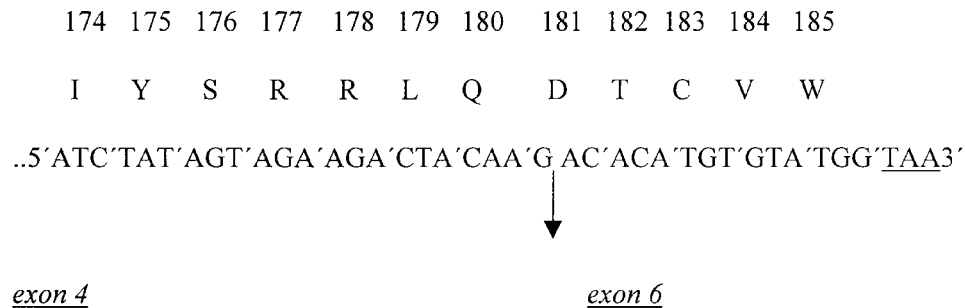
The cDNA PCR product of the patient with the IVS5+1G>A mutation showed two bands in the agarose gel electrophoresis (Figure 1), a normal sized (540 bp) and a smaller (458 bp) cDNA fragment. The larger fragment corresponded to the normal GTPCH type I mRNA with a normal borderline from exon 5 to exon 6 and is the transcript of the normal allele. The smaller fragment (458 bp) is the transcript of the mutated allele. The sequencing of this fragment showed that the complete exon 5 is missing. This result suggests that a change of bases at the 5' end of the splice site at intron 5 results in a skipping of exon 5.

The exon skipping changes the reading frame, which results in a premature stop codon (Figure 2). This mutation codes 185 amino acids (instead of 250 in the normal allele), of which 180 are normal and 5 are changed. The same results were found in the genome of the father, who carries the same mutation but has no clinical symptoms.

To show the effect of this mutation on GTPCH, we measured the enzyme activity and neopterin and biopterin concentrations in fibroblasts of the family members who carry the mutation. The range of enzyme activity found corresponds to the



**Figure 1** Amplification products with two primers ( $C_5$ , 5'-AACCAATAGAAGGA-TTGTTTC-3'; Oligo 1000, 5'-ACGTACTTACACTATTAGCA-3') separated with agarose gel electrophoresis. Lanes: 1, control; 2, patient; 3, DNA molecular weight marker V (8–587 bp)



**Figure 2** Sequence of the mutated allele. The arrow indicates the transition of exon 4 to exon 6.

**Table 1** GTP cyclohydrolase I activity and neopterin and biopterin production in cultured fibroblasts

<i>Subjects</i>	<i>GTPCH activity</i> ( $\mu$ U/mg protein)	<i>Neopterin</i> (pmol/mg protein)	<i>Biopterin</i> (pmol/mg protein)
Patient	0.2	0	65
Sister	0.6	0	64
Father	0.8	40	187
DRD patients	0.1–0.6	0–27	0–139
Controls	1.4–6.5	18–98	154–303

range of other DRD patients (Table 1). Although the enzyme activity of the father's fibroblasts was markedly reduced, the production of biopterin was in the normal range. The production of biopterin in the patient and his sister correlates with the reduced enzyme activity.

## DISCUSSION

Disturbances of the metabolism of dopamine are known to be the molecular causes of DRD. They are caused in most cases by mutations in the GTPCH gene and rarely by TH deficiency. GTPCH deficiency can be inherited both autosomal dominantly and recessively, while TH deficiency is exclusively an autosomal recessive disease. The autosomal dominant form of GTPCH deficiency is more common and in contrast to the recessive form is not accompanied by hyperphenylalaninaemia. Decreased GTPCH or TH activity results in a lowered synthesis of L-dopa and dopamine. This reduction results in muscular hypertonia and dystonic movements. Early therapy with low doses of L-dopa in combination with the decarboxylase inhibitor carbidopa leads to complete restitution; side-effects of long-term therapy are not known.

So far, more than 60 different mutations in the GTPCH gene have been found (Ichinose et al 1999; Blau et al 2000). Most of them are heterozygous point mutations changing an amino acid (missense mutation) or leading to a stop codon (nonsense mutation).

Ten mutations caused a deletion and two mutations caused an insertion with a reading frame shift and a premature stop codon. Six splice site mutations led to an exon skipping. Two mutations were located in intron 1, one in intron 2 leading to a deletion of exon 2. One mutation was found in intron 3, causing deletion of exon 4. Two mutations in intron 5 result in a deletion of exon 5.

Four patients were compound heterozygotes and four patients had a homozygous mutation of the GTPCH gene. The clinical symptoms in these patients differed from those in DRD (Blau et al 2001). All mutations of GTPCH I are listed in a recent paper (Ichinose et al 1999). The mutations we found are known mutations already described (Bandmann et al 1998; Ichinose et al 1999; Tassin et al 2000). P199L and G203R are missense mutations. They cause an amino acid exchange and thus may change the steric conformation of the protein. The M211V mutation causes a deletion of two bases, shifts the reading frame and leads to a premature stop signal (codon 249 stop); 210 amino acids are normal, while 37 are altered. As these amino acids are located at the active centre of the enzyme, the enzyme activity should be severely altered (Gütlich et al 1994; Ichinose et al 1995; Thöny et al 2000; Togari et al 1992). All members of the family carrying this mutation have symptoms of DRD. It was shown that a base exchange G to A at the 5' splicing site of intron 5, found in our patient on one allele, leads to a complete loss of exon 5. This results in a reading frame shift and a premature stop code signal. The mutated allele codes for 180 amino acids instead of 250 in the wild type; 180 amino acids are normal, the remaining 5 are altered.

This mutation was found on one allele only and is located at the 5' end of the splice site of intron 5. The patient with the splice site mutation DRD was diagnosed at the age of 3 years. The father and one sister are carriers of the same mutation but never had symptoms. The enzyme activity of the GTPCH measured in fibroblasts is reduced in all members of the family who carry the mutation. In terms of the residual enzyme activity the patient and his sister can be classified as DRD patients. The father has a residual enzyme activity that is able to produce sufficient amount of biopterin.

The results of this study show the correlation of the splice site mutation and DRD. The residual enzyme activities are borderline values, but are clearly discriminative for the diagnosis. The symptoms appear in periods of increased motoric activity in childhood and disappear at an age of lower activity. It is difficult to explain why the sister never had any symptoms while other families with higher residual enzyme activities have symptoms of DRD. Most probably, other factors in addition to GTPCH deficiency modulate the clinical outcome.

In the 27 families in which we did not find a mutation in the GTPCH I gene, three had a mutation in the tyrosine hydroxylase gene (Knappskog et al 1995; Lüdecke, Bartholomé 1995; Lüdecke et al 1995). In all of the remaining families only one member had DRD, so mutations in other genes may cause the disease, like the parkin gene (Tassin et al 2000).

## ACKNOWLEDGEMENTS

This work was supported in part by the Swiss National Science Foundation grant no. 3100-054183.98 (to N.B.) and by the Deutsche Forschungsgemeinschaft Ba 385/12-2.

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