Pre- and postnatal diagnosis of tyrosine hydroxylase deficiency

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INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) is the rate-limiting enzyme in the biosynthesis of catecholamines, and it catalyses the conversion of tyrosine to 3,4-dihydroxyphenylalanine (L-dopa) (Nagatsu et al., 1964; Blau et al., 2002). An autosomal recessive form of primary L-dopa responsive dystonia in early childhood (Knappskog et al., 1995; Lüdecke et al., 1995) and L-dopa responsive parkinsonism occurring in infancy (Lüdecke et al., 1996) were the first descriptions associated with mutations in the TH gene. TH deficiency leads to a deficit of the neurotransmitters dopamine, norepinephrine, and epinephrine, which may cause developmental delay, central and peripheral hypotonia, temperature instability, chorea, ptosis, miosis, and oculogyric crises as the main clinical characteristics (Wevers et al., 1999). However, the clinical phenotype is variable and other clinical forms have been reported (Lüdecke et al., 1996; Furukawa et al., 2001; Hoffmann et al., 2003).

The diagnosis of TH deficiency is based on the measurement of neurotransmitter metabolites and pterins in the cerebral spinal fluid (CSF). The disease is biochemically characterized by low concentrations of homovanillic acid (HVA) and 3′-methoxy-4-hydroxyphenylacetic acid (MHPG), but normal levels of 5-hydroxyindoleacetic acid (5-HIAA) (Brautigam et al., 1999). The final diagnosis is only possible by mutation analysis of the TH gene (Blau et al., 2002; Scriver, 2000). To date, 13 different mutations have been identified in the TH gene (Knappskog et al., 1995; Lüdecke et al., 1995, 1996; Van den Heuvel et al., 1998; Brautigam et al., 1999; Wevers et al., 1999; De Lonlay et al., 2000; Janssen et al., 2000; Swaans et al., 2000; Furukawa et al., 2001). Treatment with oral L-dopa plus carbidopa administered orally improves the clinical manifestations, although more recently patients with poor or no response have been described (De Lonlay et al., 2000; Hoffmann et al., 2003). Prenatal diagnosis is therefore an important issue in affected families. Since TH is not expressed in amniotic fluid cells or in chorionic villus samples, prenatal diagnosis cannot be performed biochemically, but only by mutation analysis.

In the present study, we describe a new case of the severe form of TH deficiency caused by two novel mutations in the TH gene and the first prenatal diagnosis of TH deficiency. The clinical, biochemical and genetic aspects will be discussed, as well as the options given by the availability of prenatal diagnosis.

PATIENT AND METHODS

The index patient, a girl, was born as the first child of healthy unrelated parents of Caucasian origin. The
pregnancy was complicated and at six months of gestation, the mother had to be at rest to avoid miscarriage. Oligoamnios was detected and cesarean section was carried out at eight months of gestation. Birth weight was 2.300 kg.

At five months of age, the child showed severe hypotonia, absence of head control, glossoptosis, hypersalivation, eyelid ptosis, irritability, and absence of spontaneous movements with marked delay of motor milestones. Screening for inherited metabolic disorders in blood and urine was negative. Brain magnetic resonance investigation (MRI), electromyography, nerve conduction, fundus oculi and cardiological examinations were all normal. At 10 months of age, oculogyric crises appeared. At 18 months of age, the patient had severe truncal hypotonia with ptosis, sialorrhea, oropharyngeal secretions, swallowing and feeding difficulties, and noisy breathing that caused recurrent respiratory infections. At three years of age, the patient showed the same clinical picture but with a more severe delay in psychomotor development. No diurnal fluctuation in symptoms was observed, except for the oculogyric crises, which always appeared in the afternoon. At that time, CSF was analyzed for neurometabolic disorders, including neurotransmitter and pterin metabolism defects, and the results were suggestive for TH deficiency.

Biochemical investigations

CSF was collected using a standardized protocol for lumbar puncture. Briefly, CSF samples were collected between 7:00 and 10:00 a.m., and stored at −70°C protected from light until they were analyzed. The 2nd and 3rd mL of CSF were used for neurotransmitter and pterin determinations, respectively. The neurotransmitter metabolites 5-HIAA, 5-hydroxytryptophan (5-OHTRP), HVA, MHPG and 3-O-methyl dopa (3-OMD) in CSF were quantified by ion-pair HPLC with electropherographic detection, using a previously reported procedure (Ormazabal et al., 2005). CSF neopterin (NP) and bioperin (BP) concentrations were determined by reverse phase HPLC with fluorescence detection and with manganese dioxide oxidation at acidic conditions (Ormazabal et al., 2004).

DNA extraction

Genomic DNA was prepared from peripheral blood leukocytes by means of the NaCl extraction method (Grimberg et al., 1989). For pre- and postnatal diagnosis, genomic DNA was extracted from chorion-villus and fetal tissue respectively, by use of a commercial kit (DNA Isolation Kit, Minneapolis, Minnesota, USA).

PCR amplifications and sequence analysis

All 14 exons of the TH gene were amplified by PCR and examined by denaturing gradient gel electrophoreses (DGGE) followed by sequencing of selected exons. PCR amplifications of genomic DNA were performed in a total volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2 and 0.001% (w/v) gelatine, with the addition of primers, deoxynucleotides (dNTPs) and TaqGold or AmpliTaq (PE Biosystems). DNA sequencing was carried out with the deoxynucleotide chain-termination method using [γ-32P]-labelled primers and ThermoSequenase (United States Biochemical). The PCR sequencing conditions were 50 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 7 min. The PCR sequencing products were separated on 6% denaturing polyacrylamide gels and detected with autoradiography. For PCR amplification and sequencing of exon 9 and exon 11 from genomic DNA, the primer pairs (5'-GGCTC GGCA GTGACCCGC-3'/5'-ACC CCCCAA CCGGTC-3') and (5'-TGCTCTCC CCATC TTTCA TCC-3'/5'-CACCCCAA GGCCCTGC AGG-3') respectively was used.

Mutation detection

Genetic screening for mutations in all PCR amplified exons of the TH gene by DGGE was essentially performed as previously described (Guldberg and Gütter 1993). The detailed procedure will be published separately.

Testing for maternal contamination

Testing for maternal contamination was performed by PCR amplification of 12 highly polymorphic markers located on chromosome 13, 18 or 21, separated on an ABI 3100 followed by analyzing using GenMapper (Applied Biosystems). The loci analyzed were: D13S305, D13S742, D18S386, D18S391 (all amplified using a HEX-labelled primer), D13S628, D18S499, D18S978, D21S1411 (all amplified using a NED-labelled primer) and D13S634, D18S535, D21S11, D21S1270, D21S1435 (all amplified using a 6-FAM-labelled primer) (Mann, 2004).

Ethics

Samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. For all the studies performed, written consent from the parents was obtained.

RESULTS

Biochemical investigation

Concentrations of neurotransmitter metabolites and pterins in CSF of the patient at three years of age are reported in Table 1. A profound HVA and MHPG deficiency was observed, whereas metabolites related to serotonin (5-HIAA and 5-OHTRP) and pterins were within the reference ranges.
Molecular investigations

Several isoforms exist of the human TH. Alternative splicing produces at least four different forms of mRNA. The shortest form is called hTH1. The synthesized protein encoded by hTH2 and hTH3 differ from the protein encoded by hTH1 by insertion of 4 and 27 amino acid residues, respectively, between residues 30 and 31. hTH4 differ by insertion of all 31 (4 + 27) amino acid residues (Alterio et al., 1998). All mutations identified so far are present in all the isoforms. Genomic DNA obtained from the index patient was screened for mutations in all 14 exons of the TH gene, corresponding to the isoform hTH4 by DGGE. The obtained band pattern indicated the presence of heteroduplexes only in exons 9 and 11. Subsequent sequencing of these two exons revealed two mutations, c.982C > T (p.R328W) located in exon 9 and c.1196C > T (p.T399M) in exon 11. Testing of the parents for the two mutations confirmed inheritance in trans. The test showed that the father was heterozygous for the c.1196C > T mutation and the mother heterozygous for the c.982C > T mutation. None of the mutations could be found in 200 chromosomes of control persons of European origin. Nomenclature of TH mutations is based on human mRNA type 4 (Nagatsu and Ichinose, 1991: Genbank accession number: M17589, A in the ATG start codon is numbered c.1 and the ATG start codon is numbered p.1).

A second pregnancy, a few months after the diagnosis of the index patient, led us to perform a prenatal diagnosis. It was carried out on DNA extracted from chorion-villus at the 11th week of gestation. Sequencing of exon 9 and exon 11 revealed that the female fetus was a compound heterozygote for the two mutations. Maternal contamination was excluded by PCR amplification and analyzing of a panel of highly polymorphic markers on DNA from the father, mother and the chorion-villus. Only signal from a single maternal allele was present in the DNA extracted from the chorion-villus. Abortion was then decided upon. One year after the first diagnosis, a third pregnancy ended in miscarriage at 16 weeks of gestation. DNA was extracted from the fetus and mutation analysis showed that this fetus was also a compound heterozygote for the two family-specific mutations.

Treatment

After the diagnosis was confirmed by detection of the two mutations in the TH gene, treatment was initiated with l-dopa and carbidopa. After one year of slow increment of the l-dopa doses (at present, 9.0 mg/kg/day), a clear improvement in neurological symptoms and signs was evident, especially with regard to motor development and oculogyric crises. At present, the patient (5 years old) is able to stand up with help, but mental retardation and absence of language are still present.

DISCUSSION

TH belongs to the family of aromatic amino acid hydroxylases, also comprising phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (Harayama et al., 1992; Hufton et al., 1995). These three homotrimeric hydroxylases are closely related, all containing a ferrous iron atom and utilizing oxygen and tetrahydrobiopterin as cofactors in a hydroxylizing reaction (Harayama et al., 1992). They are organized in three domains: an N-terminal regulatory domain, a catalytic domain, and a C-terminal tetramerization domain (Hufton et al., 1995). The regulatory domain enhances the substrate specificity displayed by the catalytic domain (Daubner et al., 1997). The crystal structures of the tetramers of the C-terminal fragments of rat TH (residues 156–498) and human PAH (residues 118–452) have been determined (Goodwill et al., 1997; Fusetti et al., 1998).

The catalytic domain in TH (residues 185–486) consists of 13 α-helices, six β-strands and a number of long loops. The active site consists of a cleft at the centre of the catalytic domain. The cleft is lined primarily by four α-helices: α-6, 7, 8 and 9 (Goodwill et al., 1997). The two mutations, p.R328W and p.T399M, both representing nonconservative substitutions, are located in the lining around the catalytic cleft. p.R328W is located in α-helix 6 and p.T399M in α-helix 9. Both p.R328 and p.T399 are highly conserved within the family of aromatic amino acid hydroxylases from eukaryotes (Figure 1), stressing the importance of these residues. Only in the TH gene of the trematoda, Schistosoma mansoni is the R in codon 328 replaced by the very homologous K residue. Furthermore, mutations or deletions of the corresponding residues in the PAH gene, p.R252 and p.T323, have been observed in patients with phenylketonuria (PKU). The p.R252W, p.R252Q and p.R252G mutations in the PAH gene have all been classified as severe ‘null’ PAH mutations leading to classic PKU (Guldberg et al., 1998; Güttler et al., 1999). A deletion of p.T323 has been identified in a PKU patient. This mutation most probably also causes severe PKU (PAH mutations database (http://www.mcgill.ca/pahdb)).

The clinical phenotype is variable, presentations as parkinsonism (Lüdecke et al., 1996), l-dopa responsive spastic paraplegia (Furukawa et al., 2001), or as a progressive, severe encephalopathy (Hoffmann et al., 2003) have all been reported. The time of onset vary

Table 1—CSF neurotransmitter metabolites and concentrations of pterins (nmol/L) at diagnosis (3 years of age)

<table>
<thead>
<tr>
<th></th>
<th>5-HIAA</th>
<th>HVA</th>
<th>3-OMD</th>
<th>MHPG</th>
<th>5-OHTRP</th>
<th>NP</th>
<th>BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (3 years)</td>
<td>277</td>
<td>15</td>
<td>1.7</td>
<td>n.d.*</td>
<td>4.6</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Controls (n = 16) (3–5 years)</td>
<td>106–260</td>
<td>334–570</td>
<td>0–54</td>
<td>22–46</td>
<td>4.2–18</td>
<td>9–30</td>
<td>10–30</td>
</tr>
</tbody>
</table>

* n.d.: Not detectable.
from prenatal (Lüdecke et al., 1996; Van den Heuvel et al., 1998; Wevers et al., 1999; Braütingam et al., 1999; Jansen et al., 2000; De Lonlay et al., 2000) until 2–5 years of life (Knappskog et al., 1995; Lüdecke et al., 1995; Swaans et al., 2000).

The severity of symptoms in TH deficiency seems to be linked to the residual enzyme activity. Recombinant expression in Escherichia coli, of two TH variants containing the p.Q412K and the p.L236P mutation, revealed residual activities of 15 and 1.5%, respectively (Knappskog et al., 1995; Lüdecke et al., 1996). These results are compatible with the severe phenotype of a p.L236P homozygous patient (Lüdecke et al., 1996) and the less severe phenotype of a p.Q412K homozygous patient (Knappskog et al., 1995). The TH activity is determined indirectly in vivo by measuring the dopamine metabolite HVA in CSF. In the patient homozygous for the p.L236P mutation, the HVA values was measured to 18 (Lüdecke et al., 1996), an amount comparable to the 15 measured in the present patient. A wide range of HVA concentrations in patients has been reported, ranging from undetectable HVA values to 30% of the lowest control value (Hoffmann et al., 2003).

Also the treatment efficiency with l-dopa varies. In some patients, treatment resulted in complete or substantial improvement (Knappskog et al., 1995; Lüdecke et al., 1995; Swaans et al., 2000; de Rijk-van Andel et al., 2000), while in others treatment have no or limited clinical amelioration (Bräütingam et al., 1999; De Lonlay et al., 2000; Hoffmann et al., 2003). The lack of effect is to some extent caused by the development of dose-dependent side effects even after low l-dopa medication.

There is no simple explanation for the observed differences in presentation of the disease and treatment efficiency; comparison of the location of the mutations and the degree of conservation of the affected residues did not reveal any clear differences. However, there seems to be a correlation between the time of onset and treatment efficiency. Treatment with oral l-dopa plus carbidopa improves clinical manifestations, especially in late onset forms. l-dopa responsive patients usually presented the clinical picture at an older age, from about 20 months to 7 years (Knappskog et al., 1995; Lüdecke et al., 1995; Swaans et al., 2000; de Rijk-van Andel et al., 2000). In comparison untreated patients, typically present symptoms before 7 months of age (Bräütingam et al., 1999; De Lonlay et al., 2000; Hoffmann et al., 2003).

The mutations identified in the present patient are most likely ‘null’ or close to ‘null’ mutations. The patient presented with symptoms already from the first months of life (according to information provided by the parents), and showed severe neurological symptoms at 5 months of age. The HVA levels in CSF were extremely low, less than 5% of controls. In addition, the pregnancy was complicated with a need for rest from the sixth month of gestation. A third pregnancy ended with a spontaneous interruption of what appeared to be the third affected offspring. ‘Null’ mutations in the TH gene in mice have been reported to be lethal at the late embryonic stage (Kobayashi et al., 1995). Clinically, our patient matches a severe encephalopathic phenotype. Treatment for two years has led to a clear improvement in neurological symptoms, but only the future will show if long term l-dopa therapy in our patient with a severe form will result in substantial improvement.

Screening of the TH gene for mutations in the 14 exons by DGGE usually takes approximately four weeks. In case of an urgent situation, this test can be speeded up to about two weeks. By means of DGGE it is possible to perform a prenatal diagnosis without knowing the familial mutations in advance, although the results may be inconclusive. As demonstrated in the present case, as soon as the familial TH gene mutations were identified, the prenatal diagnosis was fast, safe and easy. In practice, prenatal diagnosis will therefore only be offered once the disease-causing mutations have been established in a particular family at risk. In order
to avoid incorrect prenatal diagnosis, identification of the maternal mutation, alone or in combination with the paternal mutation, as in the present case, should be followed by testing for maternal contamination.

As there only is little experience with l-Dopa treatment in TH deficiency, and correlation between genotype, phenotype, and treatment efficiency has not yet been established, we recommend that prenatal diagnosis should be offered in all families at risk for children with TH deficiency. The availability of prenatal diagnosis gives the parents new options. They might use the result for preparation for the birth of a child with TH deficiency, or for abortion of an affected fetus. In the present case, the parents decided to interrupt the gestation of an affected fetus, since it was very probable that this fetus was also affected with a severe encephalopathy form as the index patient.

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