

RAPID COMMUNICATION

Dominant Negative Allele (N47D) in a Compound Heterozygote for a Variant of 6-Pyruvoyltetrahydropterin Synthase Deficiency Causing Transient Hyperphenylalaninemia

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Mutations in the 6-pyruvoyltetrahydropterin synthase (PTPS) gene result in persistent hyperphenylalaninemia and severe catecholamine and serotonin deficiencies. We investigated at the DNA level a family with a PTPS-deficient child presenting with an unusual form of transient hyperphenylalaninemia. The patient exhibited compound heterozygosity for the PTPS-mutant alleles N47D and D116G. Transfection studies with single PTPS alleles in COS-1 cells showed that the N47D allele was inactive, while D116G had around 66% of the wild-type activity. Upon co-transfection of two PTPS alleles into COS-1 cells, the N47D allele had a dominant negative effect on both the wild-type PTPS and the D116G mutant with relative reduction to about 20% of control values. Whereas the mother and the father had reduced enzyme activity in red blood cells (34.7% and 51.7%, respectively) and skin fibroblasts (2.8% and 15.4%, respectively), the clinically normal patient had in these cells activities at the detection limits, although PTPS-cross-reactive material was present in the fibroblasts. The specifically low PTPS activity in the mother's cells corroborated the evidence of a dominant negative effect of the maternal N47D allele on wild-type PTPS. *Hum Mutat* 13:286-289, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: hyperphenylalaninemia; tetrahydrobiopterin deficiency; 6-pyruvoyltetrahydropterin synthase; transient co-expression

INTRODUCTION

Tetrahydrobiopterin (BH₄) deficiencies are a group of recessively inherited disorders presenting with impaired biosynthesis of catecholamines and serotonin, and hyperphenylalaninemia unresponsive to dietary treatment [Blau et al., 1996b]. They are caused by mutations in one of the genes involved in the biosynthesis or regeneration of BH₄, the essential cofactor for the enzymes phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase, tryptophan-5-hydroxylase, and nitric oxide synthase [Scriver et al., 1995]. 6-Pyruvoyltetrahydropterin synthase (PTPS) deficiency (MIM# 261640), a disorder caused by the failure of the second enzyme in the biosynthesis of BH₄, is the most common form of BH₄-dependent hyperphenylalaninemia. So far, 218 cases (57% of all cases) are listed in the International BODEF Database [Blau et al., 1996a]. About 80% of all patients with PTPS deficiency present with the severe "typical" form, with char-

acteristic truncal hypotonia and increased limb tone with pronated hand posture [Ozand, 1998]. Difficulty in swallowing, oculogyric spasms, somnolence, irritability, hyperthermia, seizures, and impaired neurophysiological development are all part of the clinical picture, depending on the stage of the disease [Blau and Blaskovics, 1996].

The absence of clinical signs can identify the phenotypically "atypical" form. However, in some infants with a PTPS deficiency, neonatal hypoto-

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nia or acute but transient behavioral abnormalities, neurovegetative signs, and sleeping difficulties were noted. Conversely, patients with the "atypical" form of PTPS deficiency may appear normal in infancy but display phenotypic changes with increasing age and should be re-evaluated after infancy. At least two cases have been well documented [Ponzzone et al., 1990; Schulpis et al., 1991], and more are listed in the BIODDEF database [Blau and Dhondt, 1999], in which the cerebrospinal fluid (CSF) neurotransmitters were found to be normal in the first months of life but became progressively neurologically abnormal at 1–2 years of age, with very low CSF neurotransmitter levels.

Thus far, 28 different disease-causing mutations distributed throughout all 6 exons have been reported from 42 PTPS-deficient patients [Thöny et al., 1994; Oppliger et al., 1995, 1997; Liu and Hsiao, 1996; Hanihara et al., 1997; Thöny and Blau, 1997; Blau et al., 1999; Liu et al., 1998]. Individual mutant PTPS have been studied to various degrees, including *in vitro* studies of purified, recombinant proteins, transient expression in transfected COS-1 cells, or examination of cross-reactive material in primary skin fibroblasts [Oppliger et al., 1995, 1997].

As expected, all mutations that cause protein truncations such as a premature stop codon or internal amino acid deletions lead to unstable and enzymatically completely inactive mutant PTPS. Furthermore, most of the point mutations that cause single amino acid exchanges were shown to be unstable and/or to be significantly reduced in activity when transfected into COS-1 cells. One representative exception to this behavior of anticipated inactivity or instability was the mutant allele K129E. The K129E mutant, found in a patient with a mild form of PTPS deficiency with transient hyperphenylalaninemia, appeared to be stable in the patient's fibroblasts, although no enzymatic activity was observed. This mutant exhibited significant *in vitro* PTPS activity when assayed as purified, recombinant proteins [Oppliger et al., 1997]. However, whereas enzyme activities of most other mutants were significantly reduced when expressed in mammalian COS-1 cells, the K129E mutant was three times more active than the wild-type protein when tested in the COS-1 or the hepatoma cells Hep G2, in contrast to its inactivity in fibroblasts. Here we present the molecular analysis of a patient with a transient form of hyperphenylalaninemia due to mutations in the PTPS gene with a dominant negative allele.

RESULTS AND DISCUSSION

Patient ID#302 was born to unrelated parents of Italian origin. Newborn screening revealed a high blood phenylalanine level of 420 $\mu\text{mol/L}$. He was thought to have mild phenylketonuria (PKU) and was put on a diet. At the age of 3 weeks, his urinary pterins (1.7% biopterin) indicated a probable PTPS deficiency. Diagnosis was confirmed by the absence of enzymatic activity in the red blood cells (RBC). CSF showed marginally reduced 5-hydroxyindoleacetic acid (217 nmol/L; normal 189–1380) and normal homovanillic acid (503 nmol/L; normal 324–1379) at the age of 5 weeks (assayed by Dr. K. Hyland, Dallas, TX). Substitution therapy with L-Dopa/Carbidopa (1.8 mg/kg/day), 5-hydroxytryptophan (1.8 mg/kg/day), and BH₄ (2.2 mg/kg/day) was started. Subsequently, the dosage of L-Dopa/Carbidopa, 5-hydroxytryptophan, and BH₄ was increased to 4.8 mg/kg/day, 4.0 mg/kg/day, and 5.0 mg/kg/day, respectively, resulting in increased but still normal levels of 5-hydroxyindoleacetic acid (540 nmol/L) and homovanillic acid (915 nmol/L). At the age of 22 months, neurotransmitter enhancing medication and BH₄ were discontinued. After 2 months, the CSF neurotransmitter metabolites were still normal and the plasma phenylalanine level has remained normal over 1 year after BH₄ was discontinued. Currently, the 3.5-year-old boy is growing and developing normally. His PTPS activity in the RBCs is still very low. A more detailed record is presented in the International BIODDEF Database (<http://www.unizh.ch/~blau/biodef1.html>). In a next step, we performed molecular genetic and biochemical analysis with the patient and his parents, including PTPS enzymatic activity measurements in RBC and primary skin fibroblasts [Thöny et al., 1994], as well as cDNA and genomic DNA mutation analysis from these fibroblasts. Furthermore, in order to demonstrate potential limiting activity of mutant PTPS, BH₄ biosynthesis was determined upon *in vitro* cytokine stimulation of GTP cyclohydrolase I in cultured fibroblasts [Milstien et al., 1993]. All analyses were carried out basically as described previously with the following modifications: for sequencing PCR-amplified fibroblast cDNA, primers PTPS208 (5'-AACCCCAATAGCTATTCTCC-3') and PTPS209 (5'-AATCACGTGTTGACCTCTTA-3') were used in addition to primers PTPS7 and PTPS11 [Thöny et al., 1994; Oppliger et al., 1997]. Genomic and cDNA sequencing was performed by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer.

The results from the molecular genetic and biochemical analyses are compiled in Table 1. The patient turned out to exhibit compound heterozygosity in PTPS, by carrying A-to-G transitions at positions 139 (139A>G) and 347 (347A>G) in the corresponding cDNA [Thöny et al., 1992]. These mutations were accordingly present in exons 2 and 6 in the genomic DNA. The resulting amino acid alterations were an Asn 47 to Asp exchange (allele N47D) inherited from the mother, and an Asp 116 to Gly (allele D116G) from the father. Neither of these mutations has been observed before. PTPS enzymatic activity for heterozygous subjects in RBC and in fibroblasts was within the normal range for the father but unusually low for the mother. The relevance of this low residual PTPS activity in fibroblasts was also traced upon cytokine-stimulation of GTP cyclohydrolase I, followed by measuring BH₄ biosynthesis (Table 1). As a result, we detected BH₄ production in both parents but not in the patient, indicating that the latter has no PTPS activity. Again, BH₄ production was much lower in the mother's fibroblasts. We also performed Western blot analysis of the fibroblast extracts and found PTPS cross-reactive material in all samples, including the patient (data not shown).

To test the effect of the single amino acid exchanges in PTPS, we transfected the alleles in COS-1 cells and assayed the enzymatic activity (Fig. 1). While the D116G allele exhibited a moderate reduction to $66 \pm 20\%$ of activity relative to wild-type (100%), the N47D allele was completely inactive. Furthermore, transfection of COS-1 cells with two PTPS alleles was carried out to mimic the situation in the different family members, i.e., wild-type and N47D for the mother, wild-type and D116G for the father, and N47D plus D116G for the patient (Fig. 1). This resulted in an expected additive effect of the D116G allele in combina-

tion with wild-type PTPS ($166 \pm 36\%$). Interestingly, the N47D allele was inhibiting the activity of both the wild-type PTPS ($33 \pm 4\%$) and the D116G mutant ($35 \pm 9\%$), i.e., having a dominant negative effect over an otherwise active PTPS. Provided that the N47D is stable in vivo, these molecular analyses strongly suggested that the maternal N47D allele has a dominant negative effect, reducing the enzymatic activity of the wild-type PTPS in the mother and the D116G allele in the patient. However, besides the still unknown molecular explanations for a peripheral type of PTPS deficiency [Scriver et al., 1995], the dominant negative effect of the N47D allele, for which we here present experimental evidence, does not explain why the patient had a transient form of hyperphenylalaninemia. The only speculations we can think of with the present data would be that either (1) the expression of the (hepatic) N47D allele changed and reduced during development and liver growth, leading to a relative disappearance of the effect from the N47D allele, or (2) during the maturation process of the growing infant, the N47D allele might be inactivated or become more susceptible to degradation, eventually leading to the disappearance of this dominant negative genotype. Unfortunately, at this time we are unable to determine the expression level or enzymatic activity of PTPS in the patient's liver. Although the molecular events of such genetic or biochemical "adaptation processes" specific for the liver are not explained, they might be responsible for the unusual transient form of hyperphenylalaninemia presented.

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TABLE 1. Mutations in the 6-Pyruvoyltetrahydropterinsynthase Gene (PTS) and Biochemical Data of PTPS-Deficient Patient and His Parents

Group	Mutation (exon)	Amino acid exchange	PTPS activity in RBC $\mu\text{U/g Hb}$ (% of controls)	PTPS activity in fibroblasts $\mu\text{U/mg prot}$ (% of controls)	Intracellular BH ₄ in cytokine-stimulated fibroblasts ^a (% biopterin)
ID#302	139A>G (2) 347A>G (6)	N47D D116G	1.03 (5.9)	0.004 (0.16)	0
Mother	139A>G (2)	N47D	6.1 (34.7)	0.063 (2.76)	12
Father	347A>G (6)	wt D116G	9.1 (51.7)	0.351 (15.40)	51
Controls		wt	11–29	1.9–2.6 ^b	95

PTPS, pyruvoyltetrahydropterin synthase; RBC, red blood cells; Hb, hemoglobin; BH₄, tetrahydrobiopterin.

^a100 × biopterin/(neopterin + biopterin).

^bHeterozygote subjects for PTPS deficiency exhibit 3–15% of normal controls.

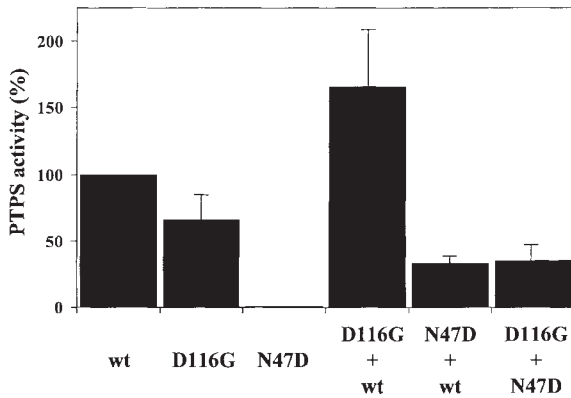


FIGURE 1. Dominant negative effect of the N47D-6-pyruvoyltetrahydropterin synthase (PTPS) allele over wild-type (wt)-PTPS and D116G-PTPS alleles in COS-1. Enzyme activities of cells transiently transfected with two pSCT1-plasmids are shown from three independent experiments. The cells contain either pSCT1 harboring a PTPS allele plus the pSCT1 vector alone, or two pSCT1-derivatives with different PTPS alleles inserted, as indicated in Methods. Polymerase chain reaction (PCR)-amplified cDNAs from patients were cloned into pSCT1 [Oppliger et al., 1997], as described previously. The pSCT1 derivatives pHSY2013, pHSY2029, and pHSY2931 were used, expressing wild-type, D116G, and N47D-PTPS, respectively. Triple transfections of COS-1 cells were performed with 2 μ g of pRSV β gal together with 4 μ g of each pSCT1 construct and the LipofectAMINE REAGENT protocol (Gibco-BRL).

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