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ORIGINAL ARTICLE

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Molecular characterization of 6-pyruvoyl-tetrahydropterin synthase deficiency in Japanese patients

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Abstract We identified three mutations in four Japanese patients with central type 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency. One missense mutation was a C-to-T transition, resulting in the substitution of Pro by Ser at codon 87 (P87S) in exon 5. Another missense mutation was a G-to-A transition, resulting in the substitution of Asp by Asn at codon 96 (D96N) in exon 5. A splicing mutation was found by skipping of exon 4 on PTPS mRNA analysis, and a G-to-A transition at the third base of codon 81 (E81E) and at the terminal base in exon 4 were detected on genomic PTPS DNA analysis. The E81E mutation affected the splice donor site of exon 4 and caused the splicing error. In COS cell expression analysis, the P87S and D96N mutant constructs revealed, respectively, 52% and 10% of wild-type activity. Patients with P87S/P87S (52%/52% in-vitro PTPS activity) exhibited 0.11 and 0 μU/g hemoglobin [Hb] in erythrocyte PTPS activity (wild-type control: 11-29 μU/gHb) erythrocyte PTPS activity, and the patient with P87S/D96N mutations (52%/10%) had 0.97 μU/gHb in PTPS erythrocyte activity. The PTPS erythrocyte activity did not coincide with the in-vitro PTPS activity based on patient genotype.

Key words 6-Pyruvoyl-tetrahydropterin synthase (PTPS) deficiency · Tetrahydrobiopterin · Mutation · Missense · Splicing · Phenotype and genotype

Introduction

6-Pyruvoyl-tetrahydropterin synthase (PTPS) is involved in the second step of the de-novo biosynthesis of 5, 6, 7, 8-tetrahydrobiopterin (BH_4) starting from guanosine triphosphate (GTP). BH_4 is an essential cofactor for hydroxylation of the aromatic amino acids, including phenylalanine in liver, but also tyrosine and tryptophan. BH_4 deficiency consists of four types of enzyme deficiencies, with PTPS deficiency the most prevalent (Scriver et al. 1995). The incidence of BH_4 deficiency in Japanese is similar to that in Caucasians, at 1 in 1,000,000 (Aoki and Wada 1988; Blau et al. 1996), whereas that in Taiwanese is much higher than in Japanese and Caucasians (Hsiao et al. 1990). BH_4 deficiency has been diagnosed in patients with hyperphenylalaninemia by mass-screening of newborns, based on BH_4 oral loading tests, analysis of urinary pterines, and determination of erythrocyte PTPS and dihydropterindine reductase (Arai et al. 1982, Shintaku et al. 1988). BH_4 deficiency if not treated causes combined symptoms of hyperphenylalaninemia and neurotransmitter deficiency, such as psychomotor retardation, progressive neurological deterioration, and red hair. Treatment of this disease consists of BH_4 to control blood phenylalanine level, and giving precursors (L-dopa/Carbidopa and 5-hydroxytryptophan) of neurotransmitters (McInnes et al. 1984; Dhondt 1984). Studies based on clinical symptoms and biochemical analysis have revealed central type PTPS deficiency with neurological symptoms, and variants such as peripheral type and partial type (Dhondt 1984; Niederwieser et al. 1987). The phenotypes of this deficiency are, thus, rather heterogeneous.

Molecular genetic study of the human *PTPS* gene began in 1991 when Inoue et al. cloned rat cDNA, and in 1992, when Thony et al. reported the sequence of the human cDNA. Thony et al. (1994) first reported homozygous mutations of R25Q and compound heterozygous mutations of R16C and 361del14 (K120 → stop) in Caucasian patients with PTPS deficiency, followed by the reports of Liu and Hsiao (1996), Liu et al. (1998), and Ashida et al. (1994) for Asians. Sixteen distinct mutations have been reported (Thony and Blau 1997). Molecular genetic analysis can provide new information on impaired PTPS function and elucidate the mechanism responsible for the clinical heterogeneity. In the present study, we analyzed the *PTPS* gene in four Japanese patients with PTPS deficiency, and identified

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two missense mutations (P87S, D96N) and one splicing mutation of exon 4.

Patients and methods

Patients

Table 1 shows the four PTPS-deficient patients studied with their biochemical phenotypes. Patient 1 is male and was born at 40 weeks of gestation in 1978, weighing 2850 g. He was the first live-born child (after one miscarriage) of second-cousin parent, who come from Honshu island. On newborn screening tests, his phenylalanine concentration was 0.97 mmol/l. He was diagnosed with hyperphenylalaninemia at another insutitution, and was treated with a low-phenylalanine diet. He developed tonic seizures, severe truncal hypotonia, mild eczema, and brown hair at 6 months of age, despite having serum phenylalanine levels of 0.24–0.36 mmol/l. The diagnosis of PTPS deficiency was confirmed by urinary pterin analysis, BH_4 loading test, and determination of PTPS activity (Table 1). Treatment was performed with a combination of BH_4, L-Dopa/Carbidopa, and 5-hydroxytryptophan. At the age of 1 year, his developmental scores were 46 for development quotient (DQ: Tsumori Inage) 47 for intelligence quotent (IQ: WISC-R); at 12 years, his social development quotient (SQ) was 77.

Patient 2 is male and was born at 39 weeks of gestation by normal delivery in 1983, weighing 3300 g. He was the fourth live-born child of unrelated parents, who come from Honshu island. His second oldest brother died of unknown causes at the age of 1 year. On newborn screening tests, the patient's phenylalanine concentration was 0.48–0.60 mmol/l. Diagnostic tests for PTPS deficiency (Table 1) were performed at the age of 1 month. He was treated with *R*-BH_4 and neurotransmitter therapy. His DQ score was 76 at 3.5 years; at 15 years, his IQ was 81 and SQ 113.

Patient 3 is male and was born at 39 weeks of gestation by normal delivery in 1986, weighing 2840 g. He was the first child of unrelated parents who come from Honshu island. On newborn screening tests, his phenylalanine concentration was 0.60–0.73 mmol/l. The diagnostic tests for PTPS deficiency were performed (Table 1) at the age of 19 days, and he was treated with high dose *R*-BH_4 monotherapy (20–30 mg/kg per day) in another institution. He developed a tendency toward irritability, truncal hypotonia, an opisthotonic posture, and poor sleep at 3 months of age. His symptoms were gradually alleviated after treatment with BH_4 and neurotransmitter precursors. His DQ score at 1 year was 115; At 5 years his IQ was 72, and at 8.5 years, his IQ was 77.

Patient 4 is male and was born at 37 weeks of gestation with severe fetal distress in 1993, weighing 2024 g. He was the first child of unrelated parents who come from Okinawa island. On newborn screening tests, his phenylalanine levels were above 0.73 mmol/l. Diagnostic tests for PTPS deficiency (Table 1) were performed at the age of 2 months, and he was treated with high dose *R*-BH_4 monotherapy (10 mg/kg per day) in another insutitution. He developed a tendency toward irritability, truncal hypotonia, and psychomotor retardation at age 5 months, and was treated with BH_4 and neurotransmitter precursors. At 1 year, his DQ was 54. Treatment was discontinued when he was aged between 1.5 and 2.5 years of age, at his parents' request. His DQ at 2.5 years was 11.

Informed consent for gene analysis was obtained from all the patients or their families.

Amplification and sequencing of PTPS cDNA

mRNA was extracted from Epstein-Barr virus-transformed cells using a quick-prep mRNA kit (Pharmacia LKB Biotechnology, Piscataway, NT, USA). For cDNA synthesis, 20 μg of total RNA was reverse transcribed using oligo(dT) 12–18 (Pharmacia) and 30 units of avian myeloblastosis virus reverse transcriptase (Life Science, St. Petersburg, FL, USA). The PTPS coding region was amplified with biotinylated sense primer Biotin-hPS1–22 (5′-GGAA-GATGAGCACGGAAGGTGG-3′) and anti-sense primer hPAS6–20 (5′-GGG-ATCAAAATCTTTTCAA-3′). Reaction mixtures contained 0.5–1.0 μg of genomic DNA, 2 mM of each dNTP, 50 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% gelatin, 50 ng of each primer, and Taq DNA polymerase. Thirty-five PCR cycles (94°C for 75 s, 50°C for 120 s, and 72°C for 180 s) were carried out, following incubation at 72°C for 5 min in a thermal sequencer (Iwaki, Funabashi, Japan). Single-strand PTPS

Table 1 Clinical and biochemical data of four studied patients with PTPS deficiency

Patient	Pterins in urine (mmol/mol creatinine)				Pterins in cetebrospinal fluid (nmol/l)				Phenylalanine (mmol/l)	PTPS activity (μU/g Hb)	Genotype
	Neo	TB	Ne/TB	Age	Neo	TB	Ne/TB	Age			
Patient 1	32.8	0.21	159	0.5 Months	51.3	7.4	6.9	7 Months	1.58	0.11	P87S/P87S
Patient 2	10.4	0.066	158	0.5 Months	46.1	3.5	13.2	0.5 Months	1.55	0	P87S/P87S
Patient 3	40.8	0.12	352	0.5 Months	211.9	19.3	11.0	0.5 Months	0.68	0.97	P87S/D96N
Patient 4	4.8	0.4	12.0	8 Months					2.96	0.18	delExon 4/ delExon 4
Control											
Newborn[a]	2.1 ± 0.52	1.1 ± 0.36	2.2 ± 1.0		22.7 ± 10.2	25.3 ± 10.0	0.98 ± 0.49				
Child[a]	1.3 ± 0.4	1.7 ± 0.5	0.8 ± 0.3						0.042–0.092		
Adult										11–29	

Values are means ± SD

Neo, neopterin; TB, total biopterin; Hb, hemoglobin; PTPS, 6-pyruvoyl-tetrahydropterin synthase

cDNA was extracted from polymerase chain reaction (PCR) products using Dynabeads M-280 (Dynal, Oslo, Norway). The single-strand DNA obtained was sequenced directly with PTPS-specific oligonucleotide primers hPAS5–18 (5′-AGAAA-GAAACTGGGCTTT-3′), hPAS3–20 (5′-TG-CAAAGTATGGCACATCCA-3′), and hPAS1–18 (5′-G-T-TTTCTTCATCACTTAG-3′) using a Sequenase DNA sequencing kit ver.2.0 (Amersham, Cleveland, OH, USA).

Genomic DNA sequencing

Genomic DNA was isolated from lymphoblasts, and exon 4 and their flanking intronic regions of PTPS genomic DNA were amplified with primers hPS2-1 (5′-ACATGGTGCTTCATGCTGAGG-3′) and hPAS2-2 (5′-TTAGAGGCCATGCGAGCAGTTC-3′) by PCR as described above. Amplified DNA was subcloned into pBluescript II SK (+) phagemid vector (Stratagene, La Jolla, CA, USA), which was transformed into competent E. Coli cells. The plasmid was purified using a plasmid preparation kit (Qiagen, Chatsworth, CA, USA), and was sequenced by a dye terminator method, using an ABI (Perkin-Elmer, Foster City, CA, USA) autosequencer.

Expression analysis

Normal full-length human PTPS cDNA was amplified with primers hPS-*Hind*III–28 (5′-AGCTCGAAGCTTGGGAAGATGAGCACGG-3′) and hPAS-*Xba*I–27 (5′-TTGAC CTCTAGATATTCCAAGGGGATC-3′) under the conditions described above. This PCR product was digested with *Hind*III and *Xba*I, and subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA, USA). Mutant PTPS cDNA was synthesized by specific base substitutions using site-directed mutagenesis. Mutant and wild-type PTPS cDNA constructs were introduced into COS cells in a mixture of 20mM hydro-xyethylpiperazine etharesulfonic acid (HEPES), pH 7.05, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, and 6mM dextrose by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) at 200V with 960-μF capacitance, as described elsewhere (Okano et al. 1994). The cells were harvested after 72h of culture, and PTPS mRNA and PTPS activity in cellular extracts were determined. PTPS mRNA levels in COS cells transfected with the normal or mutant PTPS cDNA constructs were determined by dot-blot hybridization for serially diluted total-RNA samples, using a PTPS cDNA probe labeled with [α-^{32}P]-dCTP (NEN, Boston, MA, USA) by the Megaprime DNA labeling system (Amersham).

Assay of PTPS activity

The transfected COS cells and fibroblasts were harvested after 72h and were diluted with 100μl of 0.1M Tris-HCl buffer (pH 7.4) with 10mM dithiothreitol (DTT). The samples were lysed by freezing and thawing twice and sonicated for 2min on ice. After centrifugation at 20 000*g* for 30min, the supernatants were tested for PTPS activity. Protein concentration was measured with the protein assay kit 1 (Bio Rad). PTPS activity was measured as described elsewhere (Shintaku et al. 1988). One unit of PTPS activity produced 1μmol biopterin per min under the assay conditions described. The entire study protocol was approved by the Institutional Review Board of the Osaka City University Medical School.

Results

Identification of PTPS mutation in Japanese patients

Direct sequence analysis of PTPS cDNA from the Japanese patients with PTPS deficiency revealed two missense mutations and a splicing mutation. Patients 1 and 2 exhibited a homozygous missense mutation with a C-to-T transition at nucleotide 259 of the PTPS cDNA, resulting in the substitution of serine for proline at amino acid codon 87 (P87S). The sequence gel demonstrated the presence of only one single band (T) in patients 1 and 2, while the C and T bands were present at the same position in the parents of patient 1, as shown in Fig. 1. These results indicated that patients 1 and 2 bore the homozygous P87S mutation, and that their parents were heterozygous carriers of the same mutation. Patient 3 exhibited two missense mutations. The first mutation was P87S, the same as that in patients 1 and 2. The second missense mutation was a G-to-A transition at nucleotide 286, resulting in the substitution of asparagine for aspartic acid at amino acid codon 96 (D96N), as shown in Fig. 2.

On PCR amplification of PTPS cDNA, patient 4 exhibited a shorter band (458bp) than the expected wild-type band (515bp). His parents exhibited two bands; one at 515bp, as expected, and the other at 458bp, similar to that in the patient (Fig. 3a). Skipping of exon 4 was found by sequencing the short bands of PTPS cDNA from patient 4 and his parents (Fig. 3b). Exon 4 and the flanking intronic region were amplified from the genomic DNA of this patient and his parents, followed by subcloning and sequencing. We found a G-to-A transition at nucleotide 243, resulting in no substitution of glutamic acid at amino acid codon 81, as shown in Fig. 3c. This transition was at the terminal sequence of exon 4. The parents of patient 4 were heterozygous carriers of the same mutation.

Mutation verification by expression analysis in mammalian cells

To verify that these substitutions were responsible for the PTPS phenotype, the P87S and D96N mutations and wild-type full-length human PTPS cDNA were subcloned into the eukaryotic expression vector pRC/CMV, and tranfected into COS cells. The PTPS activity of each mutant construct was calculated after correcting for the efficiency of transfection into COS cells, by determining PTPS mRNA levels by

dot-blot hybridization of serially diluted RNA from transfected cells (Table 2). The PTPS activities of the P87S and D96N mutant proteins were 52% and 10%, respectively, of the normal control PTPS activity after correcting for the efficiency of transfection into COS cells.

Discussion

We identified two missense mutations of P87S and D96N and a splicing mutation of exon 4 (E81E) in four Japanese patients with the central type of PTPS deficiency. COS cell expression analysis showed that the PTPS activities of the pRcCMV/P87S and pRcCMV/D96N constructs were decreased to 52% and 10%, respectively, of the wild-type control constructs, indicating that both P87S and D96N mutations are causes of PTPS deficiency. Human PTPS exhibits 83% homology of amino acid sequence with that of rat and 41% with that of salmon. In particuar, the sequence between valine at codon 70 and glutamic acid at codon 145 in human PTPS exhibits a high degree of amino acid homology (61%) among the three species, humans, rat, and salmon (Thöny et al. 1992). This region is therefore considered important for PTPS activity, and the P87S and D96N mutations are in this conserved region.

Patient 4 exhibited skipping of exon 4 on PTPS cDNA analysis, and a G-to-A transition (E81E) at the terminal sequence of exon 4 on genome analysis of PTPS. This mutation did not change the gt of the consensus sequence in the splicing donor site. Shapiro and Senapathy (1987) have reported a splicing score system for 5′ splice donor sites. The score at the original splice donor site at the boundary of exon 4 and intron 4 of the *PTPS* gene was 95.4, while the score of the E81E mutation was 83.0 at the same site. The E81E mutation produces loss of the splice donor site and results in the skipping of exon 4, with a reduction in mutant PTPS activity. The splicing mutations which occurred via nucleotide changes in exon regions have already been observed in mutations of fibrillin, ornithine δ-aminotransferase, phenylalanine hydroxylase, and β-hexosaminidase (Dietz et al. 1993; Okano et al. 1994; Wakamatsu et al. 1991).

The E81E (delExon 4) mutation has not previously been identified in either Asians or Caucasians. The P87S and D96N mutations have not been identified in Caucasians. The P87S and D96N mutations were frequent in Chinese patients with PTPS deficiency in Taiwan, and accounted for 36.8% and 7.9% respectively, of the alleles in PTPS deficiency. P87S is the most prevalent mutation in Taiwanese (Liu et al. 1998). These three mutations did not occur in the CpG dinucleotides, a hot spot for mutations. The frequency of PTPS deficiency in the mass-screening of newborns conducted in Taiwan was much higher than that in Caucasians and Japanese. These findings sugggest that P87S and D96N mutations had occurred in an ancestor common to the Chinese and Japanese, and thereafter, the P87S mutation was expanded in Taiwanese by the founder effect.

The erythrocyte PTPS activities in patients 1 and 2, with the homozygous P87S mutation, were 0.6% and 0% of the wild-type control activity, while the in-vitro PTPS activity of P87S was 50% of the wild-type control activity on expression analysis. The erythrocyte PTPS activity in patient 3, with P87S and D96N mutations, was 6% of the wild-type control, while P87S protein accounted for 50% of the wild-type control on expression analysis and D96N protein for

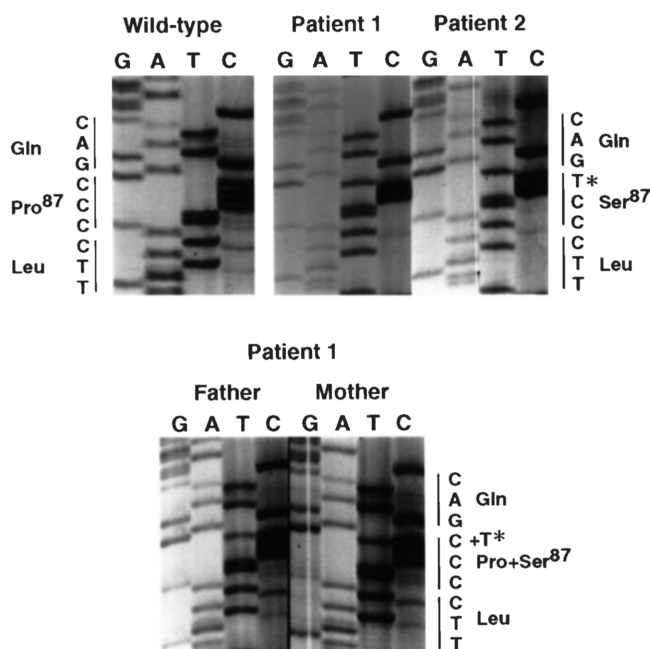

Fig. 1. DNA sequence analysis of the 6-pyruvoyl-tetrahydropterin synthase (PTPS) mutant alleles in patients 1 and 2 and the parents of patient 1. Polymerase chain reaction (PCR) products were sequenced with specific oligonucleotide primer hPSAS4–21 GTCCCA-GA-TATAAACAGCTAC. The mutant sequence exhibits a C-to-T transition at nucleotide 259 of the PTPS cDNA, resulting in the substitution of proline by serine at amino acid codon 87

Table 2 PTPS mutations in Japanese patients and PTPS activities on COS cell expression analysis

Mutation	Position	Nucleotide change	PTPS activity (μ units /μg protein)	mRNA (cpm)	Percentage of normal control PTPS activity
Mock			10.0	54	0
Normal			90.4	306	100
P87S	Exon 5	c. 259C → T	35.2	205	52
D96N	Exon 5	c. 286G → A	15.1	222	10
E81E (delExon 4)	Exon 4	c. 243G → A	—	—	—

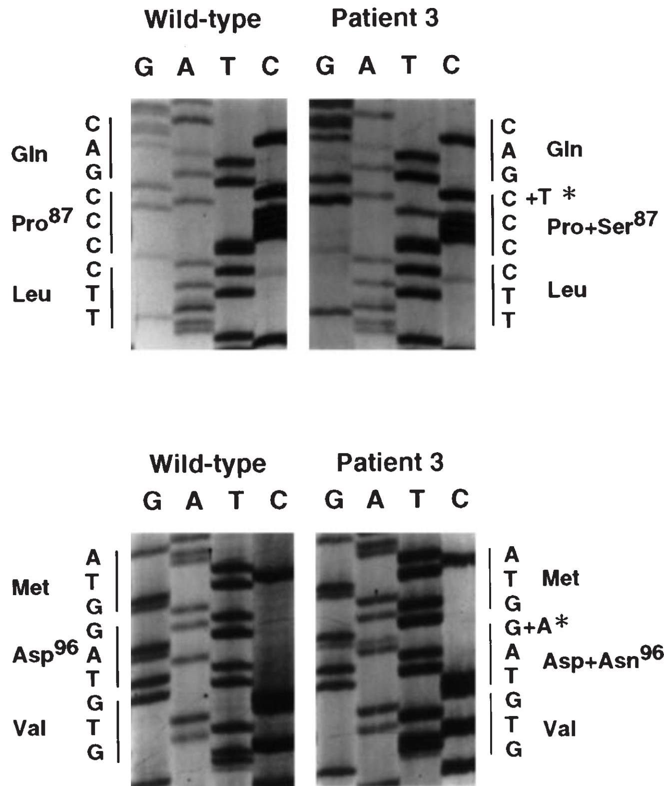

Fig. 2 DNA sequence analysis of the PTPS mutant alleles in patient 3. PCR products were sequenced with specific oligonucleotide primer hPSAS4–21 GTCCCAGATATAAACAGCTAC. The mutant sequence exhibits a G-to-A transition at nucleotide 286 of the PTPS cDNA, resulting in the substitution of aspartic acid by asparagine at amino acid codon 96

10%. Thus, the in-vitro PTPS activity on COS cell expression analysis was higher than the erythrocyte PTPS activity. Oppliger et al. (1995) reported that PTPS activity in fibroblasts from patients with the central type of PTPS deficiency with homozygous P87L mutation was less than 1% of wild-type control, while the in-vitro PTPS activity on COS cell expression analysis and in recombinant protein was 30% of wild-type control. The major cause of the high in-vitro PTPS activity is likely to be the transient overexpression of mRNA transcribed from plasmid PTPS cDNA with a strong promoter, as opposed to the steady-state expression of PTPS proteins under in-vivo physiologic control. Therefore, the processes responsible for the synthesis of these PTPS proteins in expression analysis are clearly different from those involved in the in-vivo physiologic control of the mechanisms of regulation, transcription, and splicing, and may also differ in the post-translational modification process. This overestimation of enzymatic activity in a COS cell expression system has also been found for other inherited metabolic disorders (Okano et al. 1991).

In phenylketonuria and some other congenital metabolic disorders, a correlation between genotype and clinical phenotype has been observed, and clinical symptoms have been predicted from patient genotypes (Okano et al. 1991). However, we observed no clear relationship between erythrocyte PTPS activity and PTPS activity determined by COS cell expression analysis based on genotype. The erythrocyte PTPS activity (0.97 μU/g hemoglobin [Hb]) in patient 3 was higher than those (0.11 μU/g Hb and 0 μU/g Hb) in patients 1 and 2, while the in-vitro PTPS activity (52%/10% of wild-type control) in patient 3 was lower than those (52%/52%) in patients 1 and 2. Thöny et al. (1994) also reported a patient with central type of PTPS deficiency with 0% of erythrocyte PTPS activity who exhibited higher in-vitro PTPS activity on expression analysis than a patient with peripheral type of PTPS deficiency with 13% of erythrocyte PTPS activity. Further studies, using molecular and biochemical analyses, are needed to characterize the structural and functional differences between mutant and wild-type PTPS protein, and these studies should reveal the correlation between genotype and clinical phenotype.

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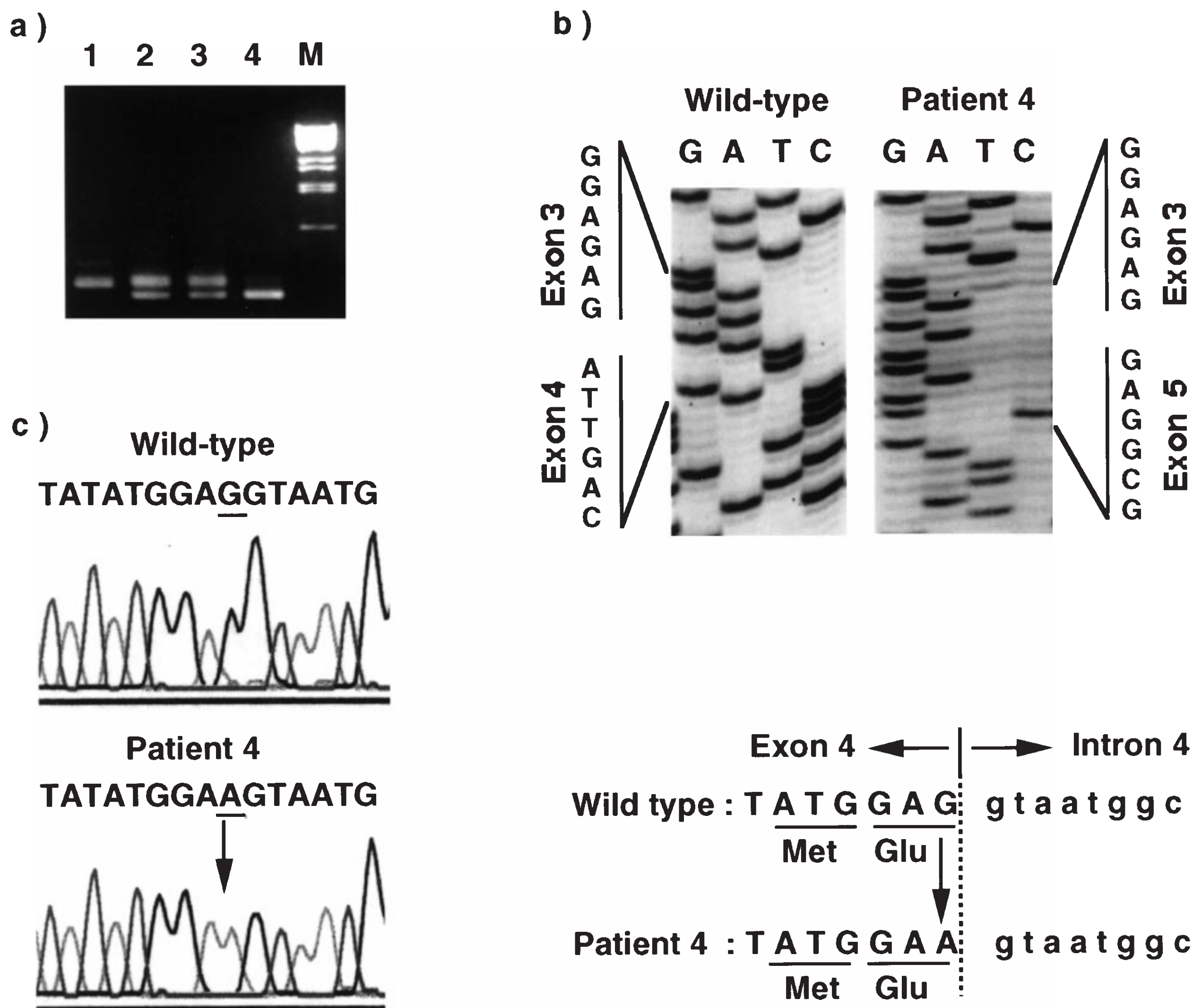

Fig. 3a-c. **a** Migration on a 2.0 % agarose gel of the PTPS cDNA of patient 4 and his parents, amplified by PCR. *Lane 1*, Wild-type control; *lane 2*, father of patient 4; *lane 3*, mother of patient 4; *lane 4*, patient 4; *lane M*, *Bst*EII digest of Lambda DNA as size marker. **b** DNA sequence analysis of the short band and expected band of PTPS cDNA in patient 4. PCR products were sequenced with specific oligonucleotide primer hPSAS4–21 GTCCCAGATATAAACAGCTAC. The sequence of the short band shows skipping of exon 4 of PTPS cDNA in patient 4. **c** DNA sequence analysis of exon 4 and the flanking intronic region in patient 4. PCR products were subcloned and sequenced with universal M13 forward and reverse primers. The mutant sequence exhibits a G-to-A transition at nucleotide 243 of the PTPS cDNA in all four clones in patient 4, resulting in no substitution of glutamic acid at amino acid codon 81

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