Homozygous Variegate Porphyria: 20 y Follow-Up and Characterization of Molecular Defect

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The long-term follow-up of a homozygous variegate porphyria patient revealed severe photosensitivity accompanied by mild sensory neuropathy and IgA nephropathy. A 35T to C transition in exon 2 (I12T) and a 767C to G transversion in exon 7 (P256R) of the protoporphyrinogen oxidase gene were identified from both alleles of the patient's cDNA and genomic DNA samples. Both prokaryotic and eukaryotic expression studies showed that the first mutation in the evolutionary conserved region resulted in a decrease in the protoporphyrinogen oxidase activity in contrast to the polymorphic substitution in exon 7, which affected the function of the enzyme assayed in *Escherichia coli* but not COS-1 cells. *Key words: mutation/porphyria/protoporphyrinogen oxidase/variegate porphyria. J Invest Dermatol 116:610–613, 2001*

Variegate porphyria (VP) is a metabolic disorder of heme biosynthesis resulting from diminished activity of protoporphyrinogen oxidase (PPOX, EC1.3.3.4) (Brenner *et al*, 1980). The disease is inherited as an autosomal dominant trait, and is characterized by photosensitivity and occasional acute neurovisceral attacks. The cDNA (1.7 kb), the genomic structure (13 exons spread over 5.5 kb), and the chromosomal localization (1q22–23) for the PPOX gene have been characterized (Nishimura *et al*, 1995; Taketani *et al*, 1995, Roberts *et al*, 1996). To date around 80 mutations have been reported in the PPOX gene.

In Finland, the prevalence of VP has been estimated to be 1.3:100,000 (Mustajoki, 1980), and to date around 100 patients drawn from a total of 19 families have been identified. In one of these families, a child was found to have a homozygous form of the disease based on the low PPOX activity (around 10% of normal) in his lymphocytes (Mustajoki *et al*, 1987). Ten other VP patients have been described with similar manifestations of childhood onset of severe photosensitivity accompanied by mental or growth retardation (Hift *et al*, 1993; Roberts *et al*, 1998). Of those, six cases have been heteroallelic (Meissner *et al*, 1996; Frank *et al*, 1998; Roberts *et al*, 1998, Palmer *et al*, 2000) and two cases homoallelic with a varying amount of residual PPOX activity (9–25%) (Roberts *et al*, 1998).

In this investigation we describe the long-term follow-up of the homozygous patient and his relatives together with characterization of the underlining molecular defect in the PPOX gene.[1]

MATERIALS AND METHODS

Patients A homozygous patient and 16 of his family members (**Fig 1*A***) and 17 other VP patients representing altogether 18 of the 19 Finnish VP families and 40 healthy unrelated Finnish controls were investigated by DNA analysis. The diagnosis for VP was based on clinical symptoms and on high fecal excretion of protoporphyrin and coproporphyrin (**Table I**) (Li *et al*, 1986), and/or low lymphocyte PPOX activity (Deybach *et al*, 1981) measured in at least one of the family members. The diagnosis of the homozygous patient was based on the low lymphocyte PPOX activity (0.4–0.6 nmol per mg protein), and his asymptomatic parents, who were first cousins, had half normal activity (2.7 and 2.3 nmol per mg protein, normal 3.6–6.0, Mustajoki *et al*, 1987).

DNA, RNA extractions and cDNA synthesis DNA was extracted from white blood cells (Higuchi, 1989) and total RNA from Epstein–Barr virus transfected lymphoblastoid cell lines (Chirgwin *et al*, 1979; Sambrook *et al*, 1989). Complementary DNA was synthesized from patients' total lymphoblast RNA by Superscript II RNase reverse transcriptase (Gibco BRL Life Technologies, MD) using random hexamers.

Sequencing of the genomic DNA and cDNA The cDNA samples were amplified using specific primers 5′-TCATTTTCTCTCATCCCTACCTA-3′ for exon 1 and 5′-CATGAATGAGAGTTGGGGATCAGCTG TTAG-3′ for exon 13, 50 pmol each in 100 μl of a solution, and 2 U of DNA polymerase (Dynazyme, Finnzymes, Finland). For sequencing the genomic DNA, the DNA (200 ng) was amplified using XL PCR Kit (Perkin Elmer, NJ) and specific primer pairs: 5′-TCATTTTCTCTCATCCCTACCTA-3′ for exon 1 and 5′-CTTCCAGCGCCCTTCTGCCTGGAG-3′ for exon 7, and 5′-CCAGTCTCTTCCAAGCTGAGCAA-3′ for exon 6 and 5′-CATGAATGAGAGTTGGGGATCAGCTGTTAG-3′ for exon 13. The temperature profile in the polymerase chain reaction (PCR) was 2 min at 94°C for the first denaturation step, followed by 30 s at 94°C and 1–4 min at 63–67°C for 30 cycles. Restriction enzymes (*Ban*I, *Msp*I) were used according to the manufacturers' instructions (New England Biolabs, MA), and the amplified samples were purified using Qiaquick PCR Purification Kit (Qiagen, CA) and sequenced using the Amplicycle Sequencing Kit (Perkin Elmer) including 5 μCi α-^{33}P-ATP (Amersham, U.K.) in each sample.

Prokaryotic and eukaryotic expression of PPOX mutations The normal and mutant PPOX alleles were expressed in *Escherichia coli* using the pUC18-vector (Pharmacia LKB Biotechnology, NJ). For the I12T mutation, the mutagenesis was performed using a Chameleon double-stranded site-directed mutagenesis kit (Stratagene Cloning Systems, CA) and a sense primer (5′-GTCGTGCTGGGCGGAGGCACCAGCGGCTTGGCCGCC-3′). For the P256R mutation, an 810 bp PCR fragment 5′-TTCGCCAGGCCTTGGCTGAGCGC-3′ for exon 7 and 5′-CATGATCTAGAGTTGGGGATCAGC-3′ were digested with *Xba*I.

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Abbreviations: PPOX, protoporphyrinogen oxidase; VP, variegate porphyria.

[1]Partly described earlier in *Hepatology* (1996) 23:1–90 (Abstr.)

Table I. Results of biochemical porphyrin analysis of VP patients[a]

	Protoporphyrin (SEM)		Coproporphyrin (SEM)		Uroporphyrin (SEM)	PBG[b] (SEM)	δ-ALA[b] (SEM)
	Red cells (250–1050 nmol/l)	Feces (< 130 nmol/g)	Feces (< 100 nmol/g)	Urine (< 230 nmol/24 h)	Urine (< 35 nmol/24 h)	Urine (< 15 μmol/24 h)	Urine (< 50 μmol/24 h)
Family 1							
Homozygous patient with severe photosensitivity, chronic neuropathy and renal failure	9501 ± 851	489 ± 49	103 ± 30	351 ± 67	173 ± 45	6 ± 2	1 ± 3
Symptom-free heterozygous patients, n = 6	260 ± 90	73 ± 32	19 ± 8	156 ± 14	18 ± 3	7 ± 2	18 ± 1
Family 2							
Heterozygous patient with 2 acute attacks[c]	783	521 ± 19	379 ± 174	213 ± 37	83 ± 37	12 ± 1	25 ± 3
Symptom-free heterozygous patient[c]	640	559 ± 75	170 ± 41	161 ± 10	28 ± 8	9 ± 2	12 ± 3

[a]Poh-Fitzpatrick (1980).
[b]PBG, porphobilinogen; ALA, aminolevulinic acid; Mauzerall and Granick (1956).
[c]At least 5 measurements in remission. Specific activity nmol/mg protein.

The *Xba*I-*Xba*I fragment was ligated as a cassette into the corresponding sites of the normal human PPOX-pUC18 or PPOX-I12T-pUC18. The normal and mutant PPOX fragments were transferred to the corresponding *Eco*RI sites of the modified pCMV1-vector, pCMV5 (Anderson *et al*, 1989). COS-1 cells were transfected with pCMV5 or the PPOX-pCMV5 or with the mutant constructs using 100 μM chloroquine and $CaCl_2$ precipitation (Sambrook *et al*, 1989). The PPOX activity of Cos-1 cells alone was monitored with (3.7 ± 0.5 nmol per mg protein) or without chloroquine and $CaCl_2$ precipitation in each series (5.4 ± 1.1 nmol per mg protein). Transfection efficiency was controlled by excluding experiments in which the wild type PPOX activity was less than a 2-fold increase compared with the background activity. 0.2 mM riboflavin-containing media was added and the transformed COS cells were incubated for 48 h before harvesting.

Assay of PPOX activity Transformed cells were lyzed with 300 μl incubation buffer [150 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), ±1% (vol/vol) Tween 20], sonicated (three times 10 s), and spun down for 5 min in a cold room, and 50 μl of the supernatant was used for the PPOX activity assay (Deybach *et al*, 1981). Protoporphyrinogen was prepared from protoporphyrin IX (Porphyrin Products, Logan, UT) using sodium amalgam reduction just before use. The protoporphyrinogen solution was diluted with 50 mM GSH incubation buffer 1:1 (vol/vol) to avoid auto-oxidation and 10 μl was used as a substrate in the reaction. The assays were carried out at 37°C in the dark. At 5, 15, and 25 min, the samples were withdrawn and mixed with 1 ml of 100 mM Tris-HCl, 1 mM EDTA, 0.1% Triton-X-100 (vol/vol), pH 8.7, and 2 mM GSH. The formation of protoporphyrin was determined by fluorescence using a Hitachi spectrofluorometer (wavelength of excitation 403 nm, emission 631 nm) and the protein concentration was determined by the method of Bio-RAD Protein Assay (Bio-Rad Laboratories, CA).

RESULTS AND DISCUSSION

Identification and expression of PPOX mutations Sequencing of the full-length cDNA and genomic DNA for PPOX from the homozygous patient demonstrated a T to C transition in exon 2 at position 35 converting isoleucine to threonine (I12T) and abolishing a *Ban*I site (**Fig 1*B***), and a C to G transversion in exon 7 at position 767 converting proline to arginine (P256R) and abolishing *Msp*I, *Hap*II, and *Hpa*II sites in both alleles. His parents, who were first cousins, and grandmothers were heterozygous for the nucleic acid changes (**Fig 1*A***). None of the 40 unrelated healthy Finnish controls had the same point mutations. Of the other 17 VP families studied, both mutations were identified in one additional family. The two families probably share a common ancestor, as they originate from the central part of Finland.

Table II. Expression of mutated protoporphyrinogen oxidase in *E.coli* and COS cells

Plasmid cell	cDNA	Mean (SEM)[a]	Range[a]	Percentage
pUC18	–	6.2 ± 1.1	1.8–13.3	0
E. coli	HPPOX	47.0 ± 3.7	21.2–71.0	100
	I12T	5.5 ± 1.3	2.2–10.4	0
	P256R	20.7 ± 3.3	3.9–35.7	35
	I12T + P256R	7.4 ± 0.9	3.8–10.7	3
pCMV5	–	3.6 ± 0.6	0.5–8.2	0
COS	hPPOX	9.8 ± 1.0	4.2–19.6	100
	I12T	4.1 ± 0.5	0.7–7.9	8
	P256R	9.5 ± 1.5	1.5–21.4	96
	I12T + P256R	3.7 ± 0.4	1.9–4.8	1

[a]At least five independent transfections and/or enzyme activity measurements.

Table II summarizes the PPOX activities of the expressed normal alleles and the I12T and P256R mutations in *E. coli* and in COS-1 cells. When crude cell extracts of plasmid-containing cultures were assayed, the normal alleles were found to have high levels of PPOX activity – a mean 7-fold increase was measured from bacterial cells and a mean 3-fold increase from mammalian cells. In contrast to the normal constructs, the I12T substitution in exon 2 resulted in a loss of function in both expression systems. Furthermore, the results did not significantly differ from those obtained from the construct including both mutations identified (I12T + P256R). The P256R substitution in exon 7, which has been identified as a polymorphism in other Western European populations (Whatley *et al*, 1999), resulted in less than half of the normal PPOX activity in the prokaryotic expression system but the activity was almost normal in eukaryotic expression. Riboflavin, which is a cofactor for PPOX, was added to the transformed COS cells and bacterial cell lysates for 48 h but no significant increase in PPOX activity was detected (data not shown). These results demonstrate that mutant alleles are transcribed and that mutant mRNAs are efficiently translated to an enzymatically impaired or active protein, and furthermore that the stability of one of the mutant polypeptides is comparable to that of the normal enzyme.

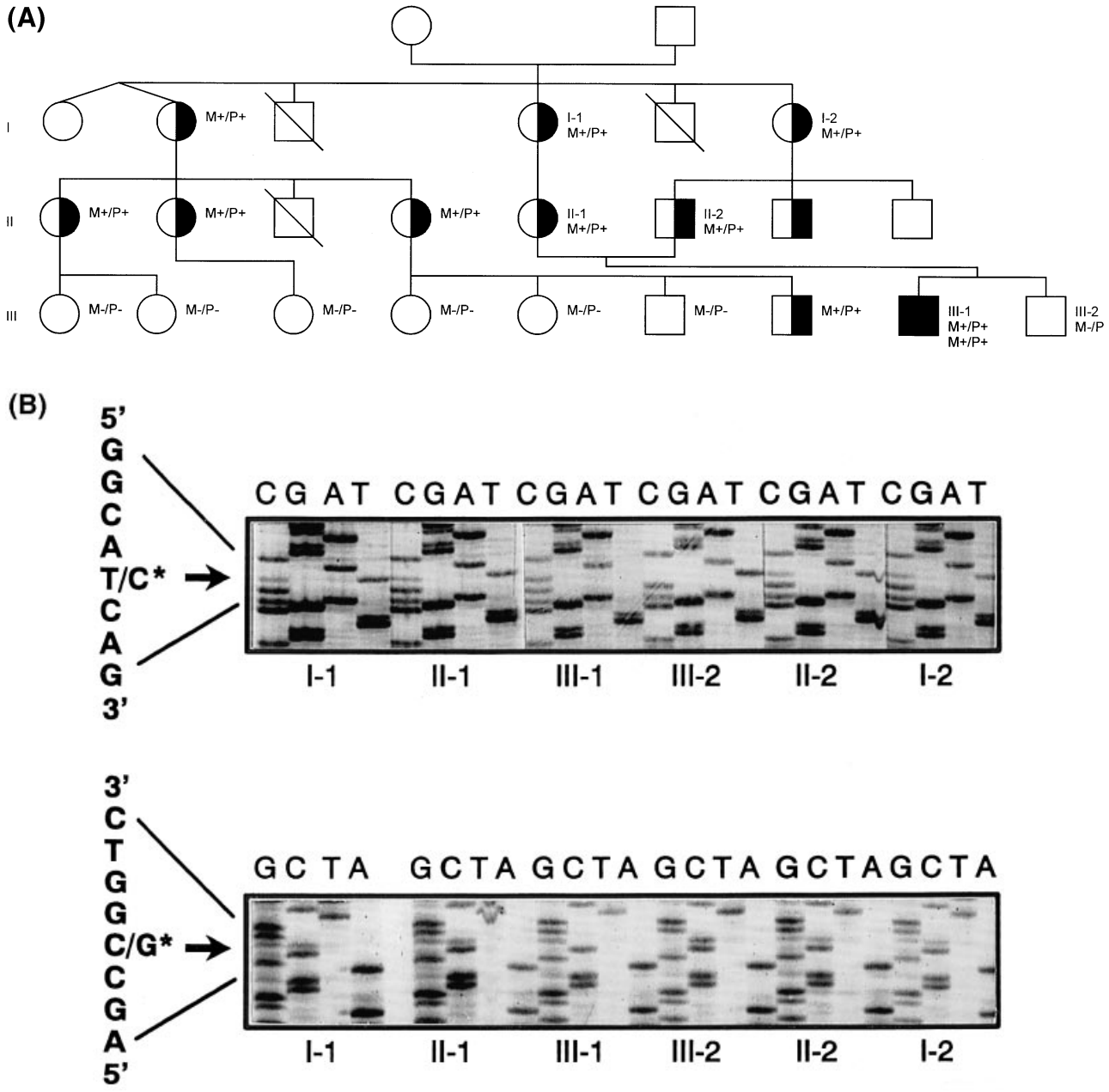

Figure 1. (*A*) Pedigree of the homozygous VP patient (■). Half black symbols are asymptomatic patients identified by mutation and polymorphic site analysis (M+/P+) or biochemical analysis or both. Healthy members of the family are identified by mutation analysis (M–/P–). (*B*) Sequencing analysis of the family members: grandmothers (I-1, I-2) and parents (II-1, II-2) are heterozygous for a 35T to C transition and a 767C to G transversion in the PPOX gene. The severely affected child (III-1) is homozygous for the mutations and his brother (III-2) has a normal sequence.

Clinical and biochemical characteristics of the homozygous patient In contrast to his asymptomatic parents, the homozygous patient developed a severe bullous skin disease post partum followed by increased fragility and keloid-like scarring. From 5 y of age he has had no severe acute photo reactions but blistering and fragility with deep erosions complicated by bacterial infections occur. Development of new scars has declined but they are still abundant including dorsal parts of the hands, ears, neck, and the scalp accompanied by partial alopecia (**Fig 2**). His fingers are markedly shortened with flexion impairment. Early closure of the phalangeal epiphyses was detected by radiographs. Mental status, EEG, and CT of the head remain normal, but sensory polyneuropathy was shown in ENMG and SEP especially in the upper extremities. Fine motor coordination disturbances are accompanied by minor verbal and visuospatial deficiencies. Raised intraocular pressure responding to a β-blocking agent and myopia were detected as early as 6 y of age (vision 0.7). Increased plasma (102 ± 27 nmol per liter, normal <3 nmol per liter) and erythrocyte protoporphyrin levels (70% zinc bound, **Table I**) were associated with severe photosensitivity and skin fragility together with microcytosis and hypochromia in his blood smear. Serum creatinine level was constantly increased (mean 130 μmol per liter, normal < 115 μmol per liter) accompanied by hematuria, proteinuria, and increase of blood pressure (RR 140/90) indicating renal failure. IgA nephropathy was confirmed in renal biopsy

The PPOX activities measured from the patient's and his mother's lymphocytes with or without riboflavin substitution were constantly low, and excretions of porphyrins and porphyrin precursors were at the previous level (data not shown).

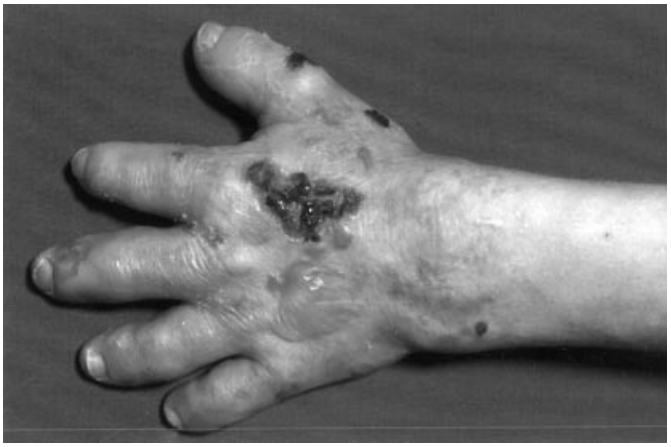

Figure 2. Severe bullous lesions, increased fragility and keloid-like scarring of exposed skin. Fingers are markedly shortened.

Riboflavin treatment did not alleviate clinical manifestations either. Although he has been exposed to potential predisposing agents such as infections, he has never experienced acute attacks indicating that the clinical manifestations are more chronic neurologic and cutaneous in origin. The heterozygous patients with I12T mutation experience no cutaneous symptoms. Only one of them has experienced two acute attacks in her youth due to use of sulfonamides, suggesting a milder phenotype of this mutation at the heterozygous state.

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