

# Identification of Mutations in the Uroporphyrinogen III Cosynthase Gene in German Patients with Congenital Erythropoietic Porphyria

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## Summary

The porphyrias are heterogeneous disorders arising from predominantly inherited catalytic deficiencies of specific enzymes along the heme biosynthetic pathway. Congenital erythropoietic porphyria is a very rare disease that is inherited as an autosomal recessive trait and results from a profound deficiency of uroporphyrinogen III cosynthase, the fourth enzyme in heme biosynthesis. The degree of severity of clinical symptoms mainly depends on the amount of residual uroporphyrinogen III cosynthase activity. In this study, we sought to characterize the molecular basis of congenital erythropoietic porphyria in Germany by studying four patients with congenital erythropoietic porphyria and their families. Using PCR-based techniques, we identified four different mutations: C73R, a well-known hotspot mutation, the promoter mutation -86A that was also described previously, and two novel missense mutations, designated G236V and L237P, the latter one encountered in the homozygous state in one of the patients. Our data from the German population further emphasize the molecular heterogeneity of congenital erythropoietic porphyria as well as the advantages of molecular genetic techniques as a diagnostic tool and for the detection of clinically asymptomatic heterozygous mutation carriers within families.

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## Key words

Porphyria • Congenital erythropoietic porphyria • Uroporphyrinogen III cosynthase • Mutation

## Introduction

The porphyrias are rare metabolic diseases, which arise from an either inherited or acquired dysfunction of specific enzymes involved in heme biosynthesis. Dysfunction of one of seven of these

enzymes results in a specific type of porphyria with a characteristic accumulation and excretion pattern of porphyrins and/or porphyrin precursors (Bickers and Frank 2003).

With only about 150 cases reported to date, congenital erythropoietic porphyria (CEP) (OMIM

263700) is an extremely rare condition and results from a decreased catalytic activity of uroporphyrinogen III cosynthase (UROS; EC number 4.2.1.75), the fourth enzyme in heme biosynthesis (Gross *et al.* 2000, Anderson *et al.* 2001, Desnick and Astrin 2002, Bickers and Frank, 2003). The enzyme is localized in the cytosol and catalyzes the conversion of the linear tetrapyrrol hydroxymethylbilane to the cyclic tetrapyrrol uroporphyrinogen III (Bickers and Frank 2003).

CEP is one of the cutaneous porphyrias and usually manifests in early childhood with diverse clinical symptoms of variable severity. Shortly after birth, cutaneous photosensitivity develops that later on can result in scarring and deformation, mainly of the hands. In the face, loss of eyebrows and eye-lashes as well as severe mutilation involving cartilage structures, e.g. the nose, is frequently observed. In milder adult-onset forms the cutaneous symptoms resemble that of porphyria cutanea tarda. (Pain *et al.* 1975). In addition to the cutaneous findings, a variable degree of hematological involvement ranging from mild forms of hemolytic anemia to intrauterine hydrops fetalis as well as splenomegaly can be found.

Biochemically, an increased excretion of uroporphyrin I and coproporphyrin I in the urine can be found. In addition, elevated excretion levels of coproporphyrin I in the stool is elevated.

In individuals suffering from CEP, UROS enzyme activity is markedly decreased but not fully absent. A total deficiency of UROS most likely would not be compatible with life as demonstrated by the lethal phenotype of homozygous UROS null mice (Bensidhoum *et al.* 1998). A reduced activity of UROS results in the non-enzymatic conversion of hydroxymethylbilane to uroporphyrinogen I. Subsequently, the four acetyl groups of uroporphyrinogen I are sequentially decarboxylated by uroporphyrinogen decarboxylase to form the coproporphyrinogen I. However, the next enzyme in heme biosynthesis, coproporphyrinogen oxidase, can only catalyze the type III isomer of coproporphyrinogen. Thus, heme metabolism can not proceed further and both uroporphyrinogen I and coproporphyrinogen I are oxidized to the non-physiological compounds uroporphyrin I and coproporphyrin I, respectively.

In CEP patients, increased levels of uroporphyrin I and coproporphyrin I accumulate in the bone marrow, skin, and several other tissues where they exert cytotoxic effects. Subsequently, exposure of the skin to ultraviolet light leads to blistering and increased

skin fragility, eventually resulting in scarring and sometimes severe mutilation.

The diagnosis of CEP can be made on the basis of a combination of typical clinical manifestations, a characteristic porphyrin excretion profile, and, in some laboratories, by measuring UROS activities in red blood cells (Tsai *et al.* 1987).

CEP is inherited as an autosomal recessive trait with apparently complete penetrance (Desnick and Astrin 2002) although one exception to this rule has been reported recently (Ged *et al.* 2004). Using a cloned cDNA, Astrin *et al.* (1991) mapped the *UROS* gene to 10q25.2-q26.3.

In families with CEP, different groups have reported a heterogeneous spectrum of mutations in the *UROS* gene (Deybach *et al.* 1990, Boulechar *et al.* 1992, Warner *et al.* 1992, Bensidhoum *et al.* 1995, Xu *et al.* 1995, Fontanellas *et al.* 1996, Tanigawa *et al.* 1996, Tezcan *et al.* 1998, Rogounovitch *et al.* 2000, Solis *et al.* 2001, Shady *et al.* 2002). Although most patients studied on the genetic level are compound heterozygotes, homozygosity for certain mutations has been encountered, preferably in consanguineous families. Of note, mutations have not only been detected in the coding regions of the *UROS* gene but also in the promoter region (Solis *et al.* 2001).

Here, we studied four unrelated CEP patients of German origin and three of their family members with PCR based techniques and identified four different mutations in the *UROD* gene. Two of these mutations are novel ones whereas the two other mutations have already been reported previously (Boulechar *et al.* 1992, Warner *et al.* 1992, Bensidhoum *et al.* 1995, Frank *et al.* 1998, Solis *et al.* 2001).

Our data further emphasize the genetic heterogeneity in CEP and the utility of molecular biological techniques in screening asymptomatic family members.

## Methods

### *Patients, controls, diagnosis, sample collection and DNA extraction*

All four index patients included in this study, among them one female and three males, were of German origin showing a severe clinical involvement including hemolytic anemia and severe photosensitivity in early life accompanied by scarring and mutilation. In each of these individuals the diagnosis of CEP was established on the

**Table 1**, Primer sequences and annealing conditions used for PCR. Note that exon 1 of the *UROS* gene is non-coding.

Amplified region	Annealing temperature	Primer sequence	Direction
Exon 2	50 °C	TTGCTTAGGAAGAGTCT	forward
		CTGTGGGATAAAGGAGTC	reverse
Exon 3	50 °C	TTT GCA AAA CCT CAG A	forward
		GTC CCT CTC TGG CTT CA	reverse
Exon 4	50 °C	CTATGGTGAAGCATTGGAA	forward
		TGCCTACTACTATTGTGAAG	reverse
Exon 5	55 °C	ATAGTTTGCTTTGCTCACA	forward
		CTGCATTCTTATCAGTAGT	reverse
Exon 6	50 °C	ATACCTGTGTATTTGCACG	forward
		CAAGAATGCACTGAGGAAA	reverse
Exon 7	55 °C	CTGAGTCCTAGAAGCAGAG	forward
		CACCCACTTCTATCACTGC	reverse
Exon 8	50 °C	AGGAGGGTGGTGCTGTATT	forward
		GGGACAGTGAAACCACATA	reverse
Exon 9	56 °C	GGCTTGAGGTCTTGATGCG	forward
		TAAGGCACCTGCTAGGCCA	reverse
Exon 10	50 °C	CAGGCTTGAGGCAGGAGTCT	forward
		AGTAACGTCCAACCGCAAAG	reverse

basis of clinical symptoms and a characteristic porphyrin excretion profile in the urine consisting of increased values for urinary uroporphyrin I and coproporphyrin I.

From the index patients and three asymptomatic family members as well as 100 unrelated control individuals of German origin blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA). All individuals provided informed consent for inclusion in this study, in accordance with guidelines set forth by the local institutional review board. Genomic DNA was isolated according to standard techniques (Sambrook *et al.* 2001).

#### PCR and mutation detection

A mutation detection strategy for the *UROS* gene was developed based on PCR amplification of all coding exons and the adjacent splice sites using primers that were designed for this study. All primer sequences and annealing conditions are described in Table 1.

PCR for amplification of the coding regions of the *UROS* gene was carried out according to the following program: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at primer specific temperatures as outlined in Table 1 for 1 min, and extension at 72 °C for

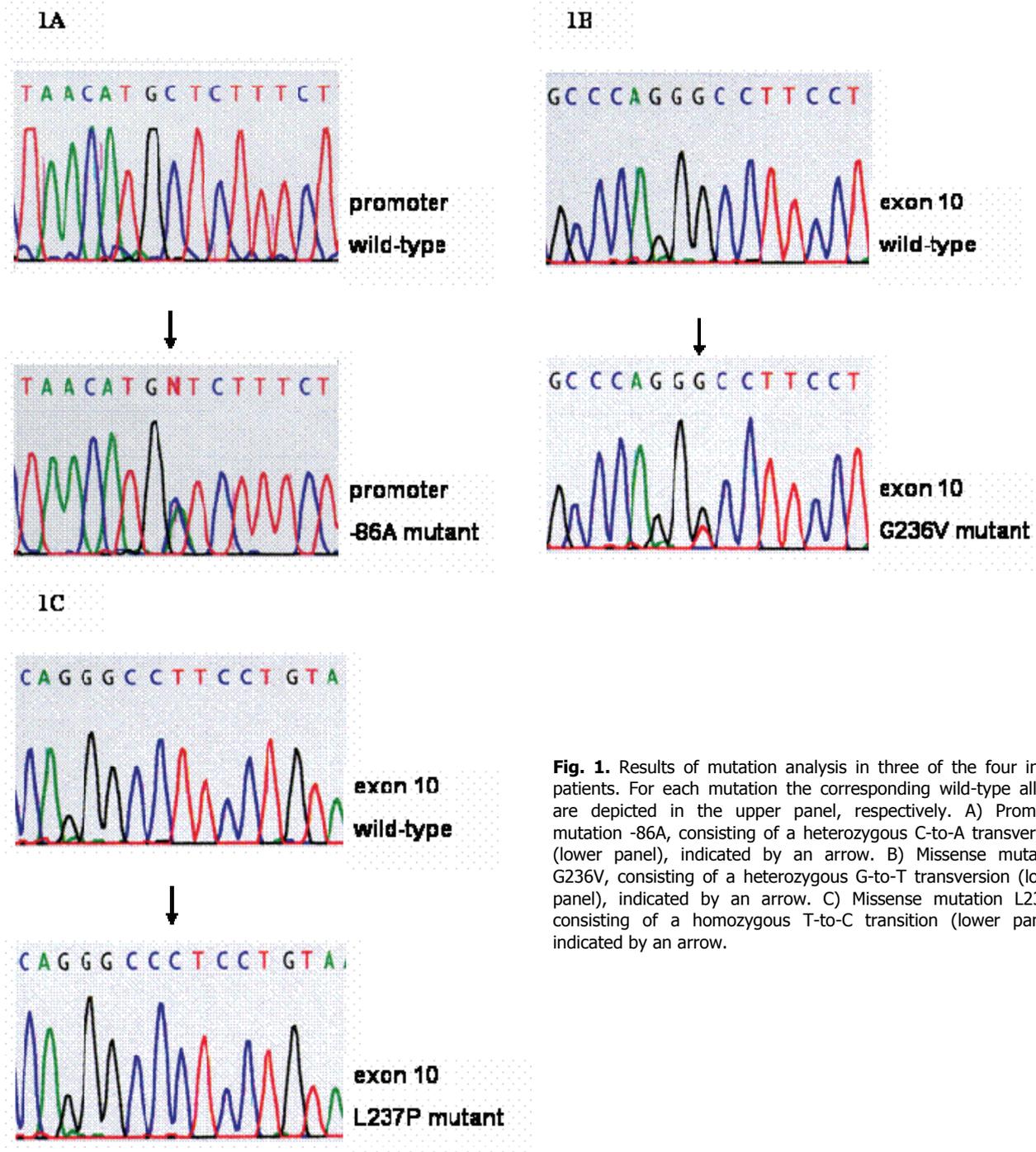
1 min and 15 s; followed by a final extension at 72 °C for 10 min, in a Biometra® TGradient thermal cycler (Whatman Biometra®, Göttingen, Germany). Each amplification reaction contained ~100 ng of genomic DNA, 50 ng/μl of each forward and reverse primer and 35 μl of Platinum Taq® PCR Super Mix (Invitrogen® Life Technologies, Karlsruhe, Germany), in a total volume of 35 μl.

Subsequently, PCR fragments were amplified with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), purified on Edge Centriflex columns (Edge BioSystems, Gaithersburg, MD), and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer from Applied Biosystems Inc. (Applied Biosystems, Foster City, CA).

## Results

#### Mutations in the *UROS* gene

Two of the mutations detected in this study, C73R (data not shown) and the erythroid promoter mutation -86A (Fig. 1A), have already been reported previously by other groups (Boulechar *et al.* 1992, Warner *et al.* 1992, Bensidhoum *et al.* 1995, Solis *et al.* 2001).



**Fig. 1.** Results of mutation analysis in three of the four index patients. For each mutation the corresponding wild-type alleles are depicted in the upper panel, respectively. A) Promoter mutation -86A, consisting of a heterozygous C-to-A transversion (lower panel), indicated by an arrow. B) Missense mutation G236V, consisting of a heterozygous G-to-T transversion (lower panel), indicated by an arrow. C) Missense mutation L237P, consisting of a homozygous T-to-C transition (lower panel), indicated by an arrow.

The two other sequence deviations encountered consisted of a G-to-T transversion at the second nucleotide of codon 236 (GGC to GTC) (Fig. 1B) and a T-to-C transition at the second nucleotide of codon 237 (CTT to CCT) (Fig. 1C), designated G236V and L237P, respectively. These two missense mutations have not been reported previously.

Two of the patients were homozygous for mutations C73R and L237P in the UROS gene, respectively. By contrast, the two other patients were

compound heterozygotes for mutations C73R and G236V as well as mutations C73R and -86A, respectively. The allelic distribution of the mutations encountered in these four patients is summarized in Table 2.

To exclude that the novel sequence deviations detected in this study were common polymorphisms we demonstrated the absence of these mutations in 200 control chromosomes of German origin by automated sequencing.

**Table 2.** Mutations detected in patients CEP 1 – CEP 4 and the respective consequences arising from these mutations. miss. mut. = missense mutation; prom. mut. = promoter mutation

Patient	Distribution of mutant alleles	Consequence of mutations
CEP 1	C73R / C73R	miss. mut. / miss. mut.
CEP 2	-86A / C73R	promot. mut. / miss. mut.
CEP 3	C73R / G236V	miss. mut. / miss. mut.
CEP 4	L237P / L237P	miss. mut. / miss. mut.

## Discussion

Here, we studied the molecular basis of CEP by screening four unrelated CEP patients and three of their relatives for mutations in the *UROS* gene. Using PCR based techniques we identified four different missense mutations, of which two have not been previously reported.

Molecular heterogeneity in CEP has been demonstrated in several previous reports in which mutations in the *UROS* gene have been identified, including missense, nonsense, frameshift, and splice-site mutations (Deybach *et al.* 1990, Boulechfar *et al.* 1992, Warner *et al.* 1992, Bensidhoum *et al.* 1995, Xu *et al.* 1995, Fontanellas *et al.* 1996, Tanigawa *et al.* 1996, Tezcan *et al.* 1998, Rogounovitch *et al.* 2000, Solis *et al.* 2001, Shady *et al.* 2002).

In the majority of the reported cases of CEP, mutations are unique to each family. As an exception to this rule, mutation C73R has been detected in approximately one-third of those patients affected with CEP from ethnically diverse populations (Boulechfar *et al.* 1992, Warner *et al.* 1992, Bensidhoum *et al.* 1995). Haplotype studies revealed that C73R is a hotspot mutation in the *UROS* gene (Frank *et al.* 1998).

The mutations detected in this study consisted of three missense mutations, designated C73R, G236V, L237P, respectively and a mutation in the erythroid promoter region, designated -86A. Two of these mutations, G236V and L237P, are novel ones.

One of the male index patients was compound heterozygous for mutations G236V and C73R. He had inherited one mutant allele from each parent, both of which were clinically unaffected. His sister harbored mutation C73R in the heterozygous state and was likewise asymptomatic. The other two male index patients were homozygous for mutations L237P and C73R, respectively. In the latter cases, no further family members were available for molecular analysis.

Regarding the patient homozygous for mutation L237P, by far the most likely reason for homozygosity would be consanguinity of his parents. The possibility of consanguineous parents has to be considered in the patient homozygous for mutation C73R, too. However, since C73R accounts for about one-third of all mutations in the *UROS* gene (Desnick and Astrin 2002) both parents could also be unrelated heterozygous carriers for C73R given the apparently high frequency of this mutation.

In CEP, a broad variation has been described regarding the extent and severity of clinical manifestations. In order to study putative genotype-phenotype-correlations prokaryotic expression studies of *UROS* proteins mutated *in vitro* have been carried out aiming at comparing the degree of clinical symptoms with the residual enzymatic *UROS* activity resulting from introduction of a specific *UROS* gene mutation. The results of these studies showed that to a certain degree the constellation of the mutations encountered in CEP and the resulting decrease in enzymatic *UROS* activity could indeed be correlated with the degree of severity of clinical symptoms observed in patients suffering from the disease (Deybach *et al.* 1990, Warner *et al.* 1992, Xu *et al.* 1995, Shady *et al.* 2002).

Interestingly, all three aforementioned patients showed a severe clinical involvement already manifesting early in infancy. This is most likely due to a dramatic reduction of residual enzymatic activity of the mutant *UROS* protein. In patients homozygous for mutation C73R this notion has already been supported by previous *in vitro* expression experiments (Warner *et al.* 1992).

Two *UROS* isoforms are encoded by the *UROS* gene on 10q25.2-q26.3, a ubiquitously expressed one and one exclusively expressed in erythroid tissues. Tissue specific expression is driven by the presence of two promoters and due to alternative splicing the two transcripts differ in their 5' untranslated regions (Aizencang *et al.* 2000). Of note, the female index patient being a compound heterozygote for the erythroid promoter mutation -86A

and mutation C73R showed a lesser degree of clinical involvement. This is in accordance with previous reports on three other patients carrying the same promoter mutation. In these patients, *in vitro* expression of the -86A allele revealed only a mild reduction of residual enzymatic UROS activity to 43 % of the normal range. If -86A was then inherited together with a null allele *in trans*, as observed in at least two patients carrying this promoter mutation, the phenotype of CEP observed was relatively mild, most likely due to the less severe reduction of residual UROS activity (Solis *et al.* 2001). Thus, the mild phenotype observed in the female index patient described herein could also be explained by the data previously presented by Solis and co-workers.

In this study, two novel missense mutations were detected, designated G236V and L237P, respectively. The glycine residue at position 236 is evolutionary conserved in human, mouse, rat, zebrafish, drosophila, *Arabidopsis thaliana*, yeast, *E. coli*, and various other bacterial species indicating a crucial role for proper protein function. Although the substitution of glycine by valine conserves the hydrophobic character of that residue it introduces a significantly larger amino acid, thereby most likely significantly altering the structure of the encoded protein.

The leucine residue at position 237 is only conserved in mouse, rat, zebrafish, and some bacteria. Other amino acids encountered at position 237 are isoleucine (e.g. in *Xenopus laevis* or *Gallus gallus*), glutamine (*Drosophila melanogaster*) and threonine (*Schizosaccharomyces pombe*), which are all large and uncharged amino acids. By contrast, the proline residue in mutation L237P is a secondary amino or imino acid in which the aliphatic side chain of the molecule is bound to the nitrogen atom of the amino group. The resulting circular structure greatly reduces the structural flexibility of the molecule and, thus, a proline substitution at this site will very likely result in an important structural alteration with subsequent disturbance of the encoded protein.

The new missense mutations detected in our study are located in close vicinity to a threonine residue at position 228. Previous *in vitro* studies revealed that this residue may play a crucial role in forming the active site of the enzyme (Mathews *et al.* 2001). This tempts us to

speculate that both aforementioned mutations could disturb the three dimensional structure close to the enzymatically active site thereby significantly disturbing proper protein function (Mathews *et al.* 2001, Desnick and Astrin 2002).

A better understanding of the molecular genetic basis of CEP is very important since there is no causal therapy for this disease. Prevention and symptomatic therapy include avoidance of ultraviolet and sunlight exposure, the use of sunscreens, and avoiding mechanical trauma. If hemolytic anemia occurs, patients may require blood transfusion therapy. At this moment, the only curative therapeutic attempt consists of bone marrow transplantation (Tezcan *et al.* 1998, Harada *et al.* 2001). One of the future therapeutic options for CEP is gene therapy by permanent transduction of hematopoietic stem cells with a vector expressing the UROS cDNA. Recent experiments using retroviral vectors expressing the UROS cDNA to transfect cultured cells *in vitro* showed a significant reduction of porphyrin load (Mazurier *et al.* 2001). However, no *in vivo* experiments have been performed so far.

Our genetic studies in German CEP patients presented herein further emphasize the molecular heterogeneity observed in this disease. Given the high frequency of mutation C73R we think that genetic counseling is mandatory, even in clinically asymptomatic heterozygous carriers. The detection of such heterozygous mutation carriers within affected families is most easily accomplished by the use of molecular genetic techniques as outlined in this study.

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