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Hepatoerythropoietic porphyria due to a novel mutation in the uroporphyrinogen decarboxylase gene

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Summary

Background—Hepatoerythropoietic porphyria (HEP) is a rare form of porphyria that results from a deficiency of uroporphyrinogen decarboxylase (UROD). The disease is caused by homoallelism or heteroallelism for mutations in the *UROD* gene.

Objective—To study a 19 year-old woman from Equatorial Guinea, one of the few cases of HEP of African descent and to characterize a new mutation causing HEP.

Methods—Excretion of porphyrins and residual UROD activity in erythrocytes were measured and compared to other HEP patients. *UROD* gene of the proband was sequenced and a new mutation identified. The recombinant UROD protein was purified and assayed for enzymatic activity. The aminoacid change mapped to the UROD protein and the functional consequences were predicted.

Results—The patient presented a novel G170D missense mutation in homozygosity. Porphyrin excretion showed an atypical pattern in stool with a high pentaporphyrin III to isocoproporphyrin ratio. Erythrocyte UROD activity was 42 % of normal and higher than the activity found in HEP patients with a G281E mutation. The recombinant UROD protein showed a relative activity of 17 % and 60 % of wild-type towards uroporphyrinogen I and III respectively. Molecular modelling showed that glycine 170 is located on the dimer interface of UROD, in a loop containing residues 167-172 that are critical for optimal enzymatic activity and that carboxyl side chain from aspartic acid is predicted to cause negative interactions between the protein and the substrate.

Conclusions—The results emphasize the complex relationship between the genetic defects and the biochemical phenotype in homozygous porphyria.

Introduction

Hepatoerythropoietic porphyria (HEP) is a rare form of porphyria that results from a deficient activity of the heme biosynthetic enzyme uroporphyrinogen decarboxylase

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(UROD)¹. The disease is caused by homoallelism or heteroallelism for mutations in the *UROD* gene (OMIM_176100) and is considered the homozygous form of familial porphyria cutanea tarda (PCT).² HEP usually presents in early childhood in contrast to PCT, which is generally manifested in adults. The cutaneous manifestations of HEP, including bullous skin lesions on light exposed areas are generally more severe and persistent than in PCT and may lead in some patients to scarring with photomutilation that resembles congenital erythropoietic porphyria (CEP, Günther disease). In comparison with CEP, HEP is not usually associated with major haematological abnormalities.³

The first HEP cases were recognized in Spain by Piñol-Aguadé et al⁴⁻⁵ and were described as a new unclassifiable form of hepatic porphyria owing to the hepatic overproduction of porphyrins and increase of protoporphyrin in blood albeit with no detectable porphyrin fluorescence in bone marrow cells. Although the disease started at early childhood it lacked some of the distinctive clinical features of CEP i.e. erythrodontia, splenomegaly or haemolysis. Subsequently, it was found that these patients presented with severe UROD deficiency⁶ due to homozygosis for a G281E missense mutation in the *UROD* gene.⁷ Enzymatic and genetic analyses of family carriers supported the view that HEP is phenotypically a homozygous form of PCT. Since the initial reports of 1969, approximately 34 cases of HEP¹ have been described. This low frequency of HEP makes it one of the rarest forms of porphyria. The genetic studies have shown that about 15 different mutations in the *UROD* gene are associated with the disease.^{3,8-9}

In this study, we report a case of HEP of African descent with a novel *UROD* mutation. Recombinant UROD with the new mutation has been purified, assayed for enzymatic activity and functional consequences were predicted.

Methods

Porphyrin analyses

Laboratory analyses of porphyrins were conducted according to EPNET (European Porphyria Network) recommendations and external quality assessment schemes. Blood, urine and stool was obtained from the patient according to Hospital protocols and protected from light. Erythrocytes were separated and frozen at -80 °C until analysis of enzymatic activity. Fluorescence emission spectroscopy of plasma porphyrins, total porphyrins in urine and faeces, erythrocyte total porphyrins and spectrophotometric determination of zinc-protoporphyrin (ZPP) and free protoporphyrin were performed according to Deacon & Elder.¹⁰

Porphyrin excretion pattern in urine and stool were characterized by reverse-phase high-pressure liquid chromatography (HPLC) according to Lim & Peters.¹¹ Separation of porphyrins and isomers was achieved using an analytical column BDS-Hypersil (Shandon HPLC, Cheshire, England). A calibration curve was set with dilutions of a mixture of carboxyl porphyrins of the I isomer series plus uroporphyrin III and coproporphyrin III (Porphyrin products Inc, Logan Utah). Identification of the four possible pentacarboxylic (5-CO₂H) porphyrin III isomers (5-bcd; 5-abc; 5- acd; 5-abd) was tentatively achieved from a pool of stool extracts of HEP patients and the HPLC reverse-phase elution order reported by Lim and Peters¹¹ Chromatograms were processed using the Waters Empower software.

Erythrocyte UROD activity

UROD activity in erythrocytes was measured according to McManus et al¹² with modifications previously described¹³. Briefly, 5-CO₂H porphyrin I (Frontier Scientific, Europe Ltd.) was reduced to 5-CO₂H-porphyrinogen (pentaporphyrinogen I) with a sodium-

mercury amalgam (Sigma-Aldrich Europe) and red blood cells lysates were incubated with pentaporphyrinogen I and the amount of coproporphyrinogen formed quantitated by HPLC.

Sequencing of the UROD loci

Since UROD activity in erythrocytes of the proband was clearly below the normal range and urinary and stool HPLC profiles suggested HEP/PCT, the UROD gene was genotyped. Genomic DNA was extracted and exons 1 to 10 of the UROD gene and the associated splice donor and acceptor sites were analyzed as previously described.¹³

Upon finding a homozygous mutation in exon 6 of *UROD* and since family members of the proband were not available for a familial segregation study, a quantitative PCR was performed in order to exclude a deletion affecting exon 6 of UROD. PCR was carried out using Power Master Mix PCR SYBR Green (Applied Bio systems, Foster City, CA, USA) following manufacturer instructions and run on an ABI 7300 PCR System (Applied Bio systems). Exon 6 of the UROD gene was amplified in DNA of the proband and in normal controls. Relative quantification was performed by a standard curve method for quantification against a control amplicon of the *GUSB* gene. Sequences for all primers used in the amplification and sequencing of *UROD* can be obtained from the authors on request.

Expression of URO-D proteins

The expression plasmid pHT77 contains the human *UROD* cDNA under the control of the T7 inducible promoter. Mutations identified in the *UROD* gene of the proband were introduced into pHT77 using the Quick-Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutations were confirmed by sequencing.

UROD proteins were expressed in Rosetta2 (*DE3*) plays (Novae, Madison, WI) in 2 L cultures by auto induction. Proteins were prepared as previously described.¹⁴ The protein concentration was determined using BCA protein reagents, (Pierce, Rockford, IL).

UROD assay

Activity of purified recombinant UROD was measured with both uroporphyrinogen-I and uroporphyrinogen-III as substrate as was previously described¹⁵. Reaction product intermediates were separated and quantified by HPLC. A 25 µL sample of the deproteinized reaction mixture was injected into a reverse phase HPLC system consisting of a Waters 2795 Separations module, a 3.9 × 300 mm µBondapak C18 column, and a Waters 474 scanning fluorescence detector. The millivolt signals detected for the various porphyrins were individually compared with those in a standard solution that contained 62.5 pmol each of uroporphyrin, 7-CO₂H-porphyrin; 6-CO₂H-porphyrin, 5-CO₂H-porphyrin, coproporphyrin and mesoporphyrin per 25-µL injection. Chromatograms were processed using the Waters Empower software.

Case Report and Results

Case report—A 19-year-old woman of African descent (Nfang ethnic group) from Bata (Equatorial Guinea) attended the Hospital Universitario Miguel Servet (Zaragoza, Spain) with a history of malaria and dermatologic complaints.

Cutaneous photosensitivity was present since she was three years old including increased fragility and blistering of the skin on sun exposed areas. A progressive scarring developed on sun exposed areas of the skin.

At examination, facial hypertrichosis and scarring areas on the upper lip, nose, hands, feet, arms and shoulders were observed. The scars were keloidal on the upper lip, arms and

shoulders. No mutilations were observed, however, scleromalacia perforans was apparent on both eyes (Figs. 1,2,3). Both hepatoand splenomegaly were noted on presentation.

Standard analyses of blood were performed at the initial examination and in a clinical follow-up. Initial analyses showed anaemia (haemoglobin 10,5 g/L, normal 12-17; packed cell volume 31,6 % , normal 36-51; mean corpuscular volume: 82,4, normal 80-100). Liver enzymes and iron homeostasis parameters were essentially normal. Lead levels in blood were also normal.

Giemsa staining of a blood smear showed *Plasmodium falciparum* trophozoites, 1-10 parasites per field. The patient was seropositive for human immunodeficiency virus (HIV), hepatitis B virus, cytomegalovirus, and parvovirus B19. Clinical history documented that HIV infection occurred when she was 17 years old. A computed tomography scan showed pulmonary consolidation of the posterolateral segment of the left inferior lobe. *Mycobacterium tuberculosis* was identified in the sputum.

The patient had seven brothers/sisters none of whom presented skin symptoms. Her parents were unrelated and no family history of porphyria existed (all were unavailable for analysis). However, the dermatologic signs in the proband suggested a congenital cutaneous porphyria and biological samples were sent to the laboratory for porphyrin analyses and DNA genotyping.

A second set of analyses were performed 14 months later after the patient had been treated for malaria and presented a recovery from anaemia (Hb 13g/L; PCV: 35%). Liver tests and iron homeostasis parameters were normal.

Porphyrin analysis

Plasma, erythrocytes, urine and stool porphyrins of the patient were analyzed at the initial clinical visit and at a clinical follow-up 14 months later. At first examination fluorescence emission scanning of plasma showed a peak at 619 nm; protoporphyrin in whole blood was increased (381 ug/dL, normal < 60) with 84% corresponding to zinc-protoporphyrin (ZPP, Table 1) and plasma porphyrins were increased to 24 ug/dL (normal < 10).

Urine showed increased amounts of total porphyrins (Table 1) with normal porphobilinogen and -aminolevulinic acid, The HPLC profiling revealed a PCT/HEP pattern with increased amounts of uroporphyrin I (30%), uroporphyrin III (10%), 7CO₂H-porphyrin (38%) and 5CO₂H-porphyrin III (15%).

HPLC profile in stool showed increased amounts of 7CO₂H-porphyrin III (13.7 %) ; 5CO₂H-porphyrin III (rings A,C and D decarboxylated, 24.8 %) and 5CO₂H-porphyrin III (rings A,B and D decarboxylated 6 %) with only slight increase of isocoproporphyrin and desethylisocoproporphyrin (figure 4). HPLC elution of the major 5CO₂H-porphyrin III isomer (rings A,C and D decarboxylated) in stool indicated identity with the major 5CO₂H-porphyrin III isomer found in urine

This pattern showed an unusually high 5CO₂H-porphyrin III to isocoproporphyrin ratio. This divergence from the characteristic HEP faecal porphyrin distribution was confirmed by analyzing in the same analytical run fresh faeces of two HEP patients with the G281E mutation⁷ (figure 4). Porphyrins in urine and blood were also compared (table 1).

All porphyrin analyses were repeated with fresh samples obtained at the follow-up clinic visit after medical treatment of the patient ; at this time the anaemia had been corrected and haemoglobin values were in the normal range. Protoporphyrin in blood was found similarly

elevated (357 ug/dL). All other results, including the elevated excretion of porphyrins in urine and faeces, were similar to those found at examination (data not shown).

UROD mutation detection

The *UROD* genomic loci were sequenced in the proband identifying a homozygous G-to-A transition at nt 509. This missense mutation results in a change from glycine to aspartic acid at residue 170 in exon 6 (G170D). The quantitation of amplified 6 excluded a possible deletion, thus confirming homozygosity for the G170D mutation in the proband. To exclude that G170D may be a common variant among Nfang ethnic group, we genotyped exon 6 of the *UROD* gene in 100 chromosomes of Nfang individuals from Equatorial Guinea (genetic material kindly provided by the department of Legal and Forensic Medicine, University of Barcelona) but none was found to harbour the G170D mutation.

UROD assay

UROD activity was assayed in erythrocytes of the proband together with erythrocytes of two HEP patients with the G281E mutation. The UROD activity in the proband was 29 U·mgHb⁻¹ thus below the cut-off value (39 U·mgHb⁻¹)¹³. However, this value represented ~42 % of the mean value of the controls and it was notably higher than that found in the HEP patients with the G281E mutation (5.2 U·mgHb⁻¹, ~ 7.5 % of normal, Table 1).

In vitro expression study

Recombinant mutant UROD protein was purified and the specific activity towards both uroporphyrinogen I and uroporphyrinogen III as substrates was determined and compared with a purified recombinant wild-type UROD. Relative activity of G170D UROD was 17.3 ± 0.6% of wild-type (mean ± standard deviation, N=2 assays) towards uroporphyrinogen I and 60.6 ± 1.1% of wild-type towards uroporphyrinogen III.

Discussion

We report a new HEP case with a mild phenotype. The first reported HEP patients, harbouring a G281E missense mutation, showed a relatively severe phenotype and the UROD enzymatic assays in erythrocytes showed a low residual activity of ~ 5-7 %. Subsequently, however, cases of HEP with higher enzyme activities in erythrocytes were reported.^{9,15-16} The new G170D mutation, also allow a considerable level of UROD activity in erythrocytes and this may be in accordance with the mild phenotype of the patient. The lack of a family history of cutaneous porphyria among residents of a very sun-drenched area of Africa suggests a relatively low clinical penetrance of this particular mutation.

It is not known if G170D in heterozygosis would induce an active phenotype. PCT, the heterozygous form UROD deficiency, is a dominant disease with incomplete penetrance³ therefore, the marginal decrement in UROD activity associated with the mutation when present in heterozygous individuals, may not have been sufficient to produce an active phenotype in the absence of other precipitating factors.

The patient presented viral infections which could be precipitating factors in PCT . However, the cutaneous symptoms had appeared in early childhood, a typical feature of HEP, while the infections occurred in adult life. Therefore, the infection cannot be considered a precipitating factor in this patient, even if the co morbidity may have aggravated the hepatic UROD deficiency

The proband presented increased ZPP in blood. This is a characteristic biochemical feature of HEP and other homozygous hepatic porphyrias and although the mechanism is not elucidated may reflect disturbed erythropoiesis secondary to the heme biosynthesis enzymatic defects.³ The ZPP increase seems to be unrelated to residual UROD activity in erythrocytes since we found in the proband an enzymatic activity within the range of some familial PCT patients,¹³ which, nonetheless, do not exhibit ZPP accumulation.

Iron deficiency may increase ZPP concentration but in this patient ZPP levels were consistently high after recovery from transient anaemia and with normal iron parameters thus indicating that ZPP increase was related to porphyria itself.

The HPLC profile of porphyrins in stool of the proband indicated striking differences between the usual HEP pattern. Interestingly, the 5CO₂H-porphyrin III to isocoproporphyrin ratio was significantly higher compared to that found in the patients with the G281E mutation. The ratio between the different 5CO₂H-porphyrin III isomers was also different. Isocoproporphyrin is formed by oxidation and metabolism by gut microflora of dehydroisocoproporphyrinogen a product of the oxidative decarboxylation of pentacarboxyl porphyrinogen III by coproporphyrinogen oxidase.³

A high proportion of 5CO₂H-porphyrin III excretion has been reported in some HEP cases,^{8,17} however the relatively low isocoproporphyrin and desethylisocoproporphyrin concentration in stool suggests either a decreased hepatic formation or a decreased biliary excretion of dehydroisocoproporphyrinogen formed from the major 5CO₂H-porphyrinogen III isomer. Unknown changes in gut microflora of this patient may also have contributed to the atypical excretion pattern.

Glycine 170 is located on the dimer interface in a loop that forms the outer edge of the active site cavity. Molecular modelling of the G170D mutation suggests that the introduced carboxyl side chain of the aspartic acid residue will be positioned pointing into the active site cleft. The carboxyl group from the aspartic acid at position 170 can be accommodated in this conformation, however, when substrate is bound the carboxyl side chain will need to flip out of the active site cleft to avoid steric clashes with propionate side chain of the tetrapyrrole. This altered interaction between the protein and the substrate may explain the observed differences in enzymatic activity between the isomer I and isomer III substrates.

Previously a case of HEP⁹ was reported in which the mutation was present at G168, in this case the mutation resulted in a glycine to arginine change. The structural perturbation of the wild type and the mutant proteins with and without product bound was minimal. However, the loop containing residues 167-172 was displaced, possibly indicating small changes in the catalytic geometry. The mutation described here, is in the same loop suggesting that the structure at this interface is critical for optimal enzymatic activity.

The new HEP patient came from an area of Africa in which no other cases of porphyria have been described to date. With the exception of South-Africa¹⁸⁻¹⁹ there are few reports of cutaneous porphyrias among indigenous Africans, the prevalence of these rare diseases and the role of precipitating factors such as malaria and viral infections being essentially unknown.

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What's already known about this topic?

Hepatoerythropoietic porphyria is a rare disease with only about 35 cases being reported. It is a homozygous form of porphyria cutanea tarda with some distinctive and intriguing features.

What does this study add?

We add a new HEP case with a novel mutation which has been analyzed by molecular modeling. Biochemical and molecular analyses are complete. Some of the biochemical features of this patient are unusual and the analysis increases our knowledge of the genotype-phenotype relationship in cutaneous porphyrias. The patient is one of the very few HEP cases reported of African descent.

Figure 1.
Scleromalacia perforans in the reported HEP patient

Figure 2.
Keloidal scars from previous bullae in the reported HEP patient

Figure 3.
Pigmented scars from previous bullae. Same lesions are observed on hands

Figure 4.

Reverse-phase HPLC profile of porphyrins in stool of the proband (homozygous G170D) compared to a HEP patient (homozygous G281E)

Total porphyrins amount: 138 nmol/g dry weight (G281E); 230 nmol/g dry weight (G170D proband); Normal < 100.

Porphyrin fractions (G281)

6III: 6CO₂H-porphyrin III (7 %);

5IIIa: 5CO₂H-porphyrin III (rings A,C and D decarboxylated, 8.5 %)

5IIIb: 5CO₂H-porphyrin III (rings A,B and D decarboxylated, 14 %)

CI = coproporphyrin I (8 %); CII = coproporphyrin III (6.6%)

DIS: desethylisocoproporphyrin (42 %); IS: Isocoproporphyrin (13.4%)

Porphyrin fractions (G170D proband)

7III: 7CO₂H-porphyrin III (13,7%); 6III: 6CO₂H-porphyrin III (2 %);

5IIIa: 5CO₂H-porphyrin III (rings A,C and D decarboxylated, 24,8%)

5IIIb: 5CO₂H-porphyrin III (rings A,B and D decarboxylated, 6 %)

CI = coproporphyrin I (24.7%); CII = coproporphyrin III (6.5 %)

DIS: desethylisocoproporphyrin (4.9%); IS: Isocoproporphyrin (5.4%)

P: Protoporphyrin (6.6 %)

Figure 5. Purified URO-D

Wild type (WT rhUROD) and G170D mutant (G170D rhUROD) URO-D were purified to homogeneity using Ni²⁺-NTA resin. A 2 µg sample of each of the proteins was separated on a 12% SDS-PAGE gel. The proteins are predicted to have a molecular weight of 43.3 kDa. Molecular weight standards are run in lane 1, the masses of the standards are labeled to the left as kDa.

Figure 6. Interaction of UROD with the product

The structure of UROD (1RY3) in complex with the product coproporphyrinogen III is shown. The location of the glycine to aspartic acid change is shown in red. The location of the residues at position 170 are approximately 8 Å apart across the dimer interface (one monomer backbone in green, the second monomer backbone in sky blue). The hydrogen bonding interaction between the carboxylate side chain of the mutation and the reaction product, coproporphyrinogen (orange), are shown as dashed yellow lines.

Table 1

Total porphyrins in urine, protoporphyrin in blood and uroporphyrinogen decarboxylase activity in erythrocytes of the proband (G170D) compared to two already reported HEP patients with the G281E mutation in the UROD gene. Urinary total porphyrins : nmol/mmol creatinine, (normal < 35) ; UROD activity : U·mgHb⁻¹ (cutoff > 39). Protoporphyrin in blood : µg/dL (normal < 60) . ZPP: zinc-protoporphyrin proportion (%) of total protoporphyrin in blood

	Urine porphyrins	UROD activity	Protoporphyrin	ZPP (%)
G281E (a)	1389	5.1	150	77
G281E (b)	1213	5.3	185	81
G170D	840	29.0	381	84