Clinical, Biochemical, and Genetic Study of 11 Patients With Erythropoietic Protoporphyria Including One With Homozygous Disease

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Objective: To study the mutations in the ferrochelatase gene (FECH) and the phenotypic expression of erythropoietic protoporphyria (EPP) in a group of Spanish patients.

Design: Case series.

Setting: University-based hospital.

Patients: Eleven unrelated patients with EPP and 19 asymptomatic relatives from 10 families.


Results: FECH gene mutations were found in all 11 patients. Ten were heterozygous and carried the IVS3-48C low-expression allele. Three novel mutations were found: IVS4/H11001delG, 347-351delC, and 130_147dup18. One patient did not present the IVS3-48C polymorphism and was found to harbor a novel A185T missense mutation in both alleles. The familial study confirmed a recessive mode of inheritance of the disease. The A185T mutation showed a residual activity 4% of normal when expressed in E. coli. This patient presented cutaneous photosensitivity similar to the heterozygous cases, but a higher protoporphyrin accumulation in erythrocytes, microcytic anemia, and early signs of liver engagement. FECH mutations were found in 10 healthy relatives, none of whom carried the low-expression allele. The frequency of the IVS3-48C allele among 180 nonporphyric Spanish individuals was 5.2%.

Conclusions: These findings confirm, among a group of Spanish patients, that most cases of EPP result from the coinheritance of IVS3-48C and a mutation in the FECH gene, and also document the existence of patients with mutations in homozygosity that may present a more severe form of the disease.

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ERYTHROPOIETIC PROTOPORPHYRIA (EPP) (Mendelian Inheritance in Man 177000) is an inherited disorder of porphyrin metabolism caused by a partial deficiency of ferrochelatase (FECH) (European Community 4.99.1.1), which catalyzes the insertion of iron into protoporphyrin IX to form heme. Patients with EPP accumulate free protoporphyrin mainly in erythropoietic tissues, but the metabolite may also reach the skin, absorb light at certain wavelengths, and generate free radicals, resulting in phototoxic cell injury. Most patients with EPP develop solely photosensitivity commencing in childhood, but in some cases liver complications also appear. The strong hydrophobic nature of the molecule does not allow renal clearance but only bile secretion. Therefore, massive hepatic accumulation of protoporphyrin in the liver may cause cholestasis and liver damage in a minority of patients with EPP. Hepatic involvement may vary in severity, ranging from abnormalities in 20% of the cases to cirrhosis and liver failure in less than 5%.1,3 Blood disorders such as mild anemia with hypochromia and microcytosis may also be detected in 20% to 50% of these patients.2,4,5

See also pages 1153, 1194, and 1209

The FECH gene is assigned to chromosome 18q21.3 (GenBank No. D00726), with more than 110 different mutations having been described in EPP families to date.6 Individuals who are heterozygous for these mutations remain asymptomatic, despite having FECH activity reduced by about 50%. Clinical expression of the disease in most cases results from
the coinheritance of a null FECH allele plus a wild-type low-expressed allele, which further reduces FECH activity to below a critical threshold of about 35%. In a haplotype segregation analysis, it was found that the low-expression allele is the C variant of an intronic single-nucleotide polymorphism (SNP) IVS3-48T/C in intron 3 of the FECH gene. Because the IVS3-48C allele is relatively common among European populations, overt EPP is normally inherited in such a way that it resembles an autosomal recessive disease with low clinical penetrance.

Erythropoietic protoporphyria is rarely inherited as an autosomal recessive disease with true mutations in both alleles. In some areas of southern Europe, the prevalence of EPP and the genetic defects underlying the disease are not sufficiently elucidated. Therefore, we investigated the mutations in the FECH gene and the phenotypic expression of the disease in a series of Spanish patients with EPP. The molecular, biochemical, and clinical study included a rare case of recessive EPP.

### METHODS

#### PATIENTS

We studied 11 unrelated patients (6 female and 5 male, with a mean age of 12.8 years [range, 4-26 years]) who attended the Department of Dermatology of the Hospital Clinic of Barcelona (Barcelona, Spain) for photosensitivity and suspicion of porphyria. The diagnosis of EPP was established initially by clinical and biochemical criteria and was later confirmed by DNA analysis of mutations in the FECH gene. A complete hematological and biochemical profile and specific liver function tests were performed in every case. In addition, 19 asymptomatic relatives from 8 families were also included in the genetic study. The study was conducted in accordance with the Declaration of Helsinki principles and was approved by the hospital ethics committee of the Hospital Clinic of Barcelona. Written consent was obtained from all patients.

#### PORPHYRIN DETERMINATION

Protoporphyrin concentration in erythrocytes was determined fluorometrically after solvent partition according to the method described elsewhere. Protoporphyrin in feces was extracted according to Lockwood et al and was analyzed by high-performance liquid chromatography according to Lim and Peters. Porphyrins in urine were analyzed by standard methods of high-performance liquid chromatography.

#### LYMPHOCYTE FECH ENZYME ASSAY

FECH activity in lymphocytes was determined by incubating a homogenate with zinc and mesoporphyrin and measuring zinc-mesoporphyrin formation by fluorometry according to the method of Rossi et al, as modified by Gouya et al. FECH activity was expressed as nanomoles of zinc-mesoporphyrin formed per hour per milligram of protein at 37°C.

#### DNA ANALYSIS

We analyzed exons 1 to 11 of the FECH gene and the associated splice donor and acceptor sites. The primers used for exon amplification are available from the authors. All polymerase chain reaction (PCR) analyses were carried out using the PCR Master Mix (Promega Corporation, Madison, Wisconsin) following the manufacturer’s instructions. The following PCR conditions were applied: initial denaturizing step (5 minutes at 95°C), 35 cycles (1 minute at 95°C, 1 minute at 57°C, and 1 minute at 72°C), followed by final extension (10 minutes at 72°C) and maintenance at 4°C until single-strand conformational polymorphism or sequencing studies were carried out. Part of intron 3 was sequenced in all samples to test for the IVS3-48C polymorphism.

Single-strand conformational analysis was used to detect mutations. Three microliters of denatured PCR product was combined with loading buffer and loaded into GeneGel Excel 12.5 acrylamide gels (Amersham Biosciences, Uppsala, Sweden) and run following the manufacturer’s instructions. The different migrations were silver stained. DNA samples with abnormal migrations were sequenced. The PCR products were purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an ABI3100 automatic sequencer (Applied Biosystems).

#### PROKARYOTIC EXPRESSION OF MUTANT FECH

Mature ferrochelatase was expressed in Escherichia coli strain JM109 (Promega Corporation) using the expression vector pKK223-3 (Pharmacia Biotech Inc, Piscataway, New Jersey). The wild-type human ferrochelatase complementary DNA (cDNA) was synthesized by PCR excluding the region encoding the mitochondrial leader sequence and including a new ATG start site of translation, and cloned into the EcoRI-HindIII sites of pKK223-3. This construct was designated pKK-FECH-wt. To generate the mutant construct pKK-FECH-A185T, a fragment of FECH cDNA, containing the A185T mutation, was generated by PCR site-directed mutagenesis using the pKK-FECH-wt plasmid as templated. Then, the PCR product was digested with the restriction endonucleases MjeI and Cvel (New England Biolabs, Beverly, Massachusetts) and the MjeI-Cvel fragment was ligated as a cassette into the corresponding sites in pKK-FECH-wt, and transformed in E coli strain JM109. The integrity of each expression construct was checked by automated sequencing. The primers are available from the authors.

Bacterial clones, each of them containing one of the plasmids pKK223-3, pKK-FECH-wt, or pKK-FECH-A185T, were grown to log phase and were induced with 5mM isopropylthiogalactoside for 3 hours. Then, cells were harvested by centrifugation and washed twice in phosphate-buffered saline. The pellets were resuspended in 230 µL of lysis buffer (100mM Tris hydrochloride, pH 7.6; 0.1% Triton-X 100) and sonicated on ice. After centrifugation, ferrochelatase activity was measured in the supernatant using protoporphyrin IX and zinc sulfate as substrates, according to the method described by Camadro and Labbe.

### RESULTS

#### CLINICAL CHARACTERISTICS

The main clinical and biochemical outcomes of the patients with EPP are given in the Table. All presented acute cutaneous photosensitivity, with the symptoms beginning in early childhood in most cases. Only 3 cases presented with chronic skin thickening over the knuckles. Microcytic anemia was present in 4 cases. Liver func-
Molecular analysis of 19 asymptomatic relatives from patients, except patient 1 who was homozygous for IVS3-48C/T, were heterozygous for IVS3-48C allele. All of these relatives had normal values of protoporphyrin in blood. Analysis of 180 nonporphyric unrelated Spanish individuals showed that the IVS3-48C allele was present in 10.5% (allele frequency, 5.2%).

**REPORT OF A CASE**

Patient 1 was a 15-year-old girl with microcytic anemia since she was 4 months old and photosensitive dermatitis since she was 4 years old. When she was 10 years old, alterations in liver function had been detected, with the persistence of microcytic anemia. She presented an itchy erythema and edema on the hands and face after sun exposure. On examination, pitted and linear scars on the nose, cheeks, and forehead and mild skin thickening over the knuckles were observed. There was no family history of photosensitivity and no apparent familial consanguinity.

Results from porphyrin analyses included the following: erythrocyte protoporphyrin level, 32 µmol/L (reference value, <1.5 µmol/L); >90% free protoporphyrin; and fecal protoporphyrin level, 1066 nmol/g of dried feces (reference value, <100 nmol/g), which allowed for the diagnosis of EPP. The excretion of coproporphyrins in urine was normal. Results from biochemical analysis included the following: aspartate transaminase level, 52 U/L (reference value, 15-200 ng/mL) (to convert to picomoles per liter, multiply by 0.179); ferritin level, 28 ng/mL (reference range, 50-150 µg/dL) (to convert to micrograms per liter, multiply by 2.247); and soluble transferrin receptor level, 0.089 mg/dL (reference range, 0.083-0.176 mg/dL) (to convert to micromoles per liter, multiply by 0.0123). The appearance of the liver on echography was normal. A hepatic biopsy revealed normal liver dysfunc-

### Table. Major Clinical and Biochemical Features and FECH Genotypes in 11 Patients With EPP

<table>
<thead>
<tr>
<th>Patient No./ Sex/Age at First Symptoms, y</th>
<th>Hemoglobin, 12-17 g/dL</th>
<th>MCV, 80-100 fL</th>
<th>Liver Dysfunction</th>
<th>Erythrocyte PP &lt;100 nmol/g of Dried Feces</th>
<th>FECH Mutation</th>
<th>IVS3-48C/T SNP</th>
</tr>
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<tr>
<td>1/F/4</td>
<td>11.5</td>
<td>73</td>
<td>Yesa</td>
<td>32</td>
<td>1066</td>
<td>A185T/A185T</td>
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<td>12</td>
<td>ND</td>
<td>757del5</td>
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<tr>
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<td>13.7</td>
<td>80</td>
<td>No</td>
<td>11</td>
<td>90</td>
<td>IVS4 +1delG</td>
</tr>
<tr>
<td>4/M/1</td>
<td>13.4</td>
<td>79</td>
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<td>10</td>
<td>715</td>
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<td>5/F/6</td>
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<td>6/M/3</td>
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<td>79</td>
<td>No</td>
<td>7</td>
<td>186</td>
<td>P334L</td>
</tr>
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</table>

Abbreviations: EPP, erythropoietic protoporphyria; MCV, medium corpuscular volume; ND, not done; PP, protoporphyrin; SNP, single-nucleotide polymorphism.

SI conversion factor: To convert the hemoglobin to grams per liter, multiply by 10.

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architecture and the presence of brown material without birefringence at polarized light. Hemoglobin electrophoresis demonstrated normal percentages of hemoglobin A, and F, and findings from DNA analysis excluded the thalassemia trait. Bone marrow examination showed normal percentages of the hematopoietic cell lineages with dysplasia signs in the erythroid cells. The bone marrow karyotype was 46,XX[20].

Findings from a molecular study of the DNA showed that this patient was apparently homozygous for a novel A185T missense mutation and homozygous for the T variant of the IVS3-48C/T polymorphism. The residual activity of the mutant FECH in E. coli was 4% of normal activity. The FECH activity in peripheral lymphocytes was 0.4 nmol of zinc-mesoporphyrin per hour per milligram of protein at 37°C (reference value, mean ± SD, 4.5 ± 1). Two years later, the erythrocyte protoporphyrin level had increased to 77 µmol/L (fecal protoporphyrin level, 1221 nmol/g of dried feces).

Familial investigation showed that both parents and a sister were heterozygous for the A185T mutation, and none harbored the low-expression IVS3-48C allele, thus confirming recessive EPP. All were asymptomatic and had an erythrocyte protoporphyrin concentration of less than 1.5 µmol/L.

**COMMENT**

We present the clinical, biochemical, and molecular characteristics of 11 cases of EPP and 19 asymptomatic relatives from 10 Spanish families. Among the series of patients with EPP, 1 had homozygous disease. Recessive inheritance of EPP, with patients presenting mutations in both alleles is rare. Only 13 cases (12 families) have been reported to date,6,9,12 most being compound heterozygotes. To our knowledge, the patient who was homozygous for an A185T mutation, and none harbored the low-expression IVS3-48C allele, thus confirming recessive EPP. All were asymptomatic and had an erythrocyte protoporphyrin concentration of less than 1.5 µmol/L.

It has been suggested that recessive EPP forms may have a higher risk of liver failure than the more usual dominant forms.10 According to published reports, severe liver disease has been observed in 4 of 13 patients with recessive EPP detected to date.6,10,12 This is a high proportion, taking into account that liver failure appears in only in less than 5% of patients with EPP dominant forms.6,25

The determinants of liver injury in EPP are not well understood because genetic factors may modulate the accumulation of protoporphyrin in the liver.26 It is likely, however, that the amount of residual FECH activity in cells12,25 may play a key role in determining the course of the disease. Most of the missense mutations found in heterozygous EPP cases show a drastic reduction or even undetectable activity when expressed in prokaryotes.27-29 In contrast, in a report of recessive EPP, all the mutations retained a FECH residual activity ranging from 12% to 50% when expressed in E. coli.12 This is in accordance with the general view that homozygous cases of porphyria must retain a substantial residual enzyme activity to be compatible with life. The A185T mutation, however, showed only 4% of the normal activity in E. coli, thus suggesting a severe impairment in functionality. Consequently, even if this homozygous patient is free of cholestasis signs and the liver markers remain stable to date, the progression of the disease should be considered of risk and advocates close clinical follow-up.

Except for the recessive case, all the other patients in our study showed mutations in heterozygosity within the FECH gene. Some of the new mutations can also cause severe impairment in enzyme activity. The c.351delC mutation is a single C deletion from a stretch of 5 C that causes a frameshift and introduces a premature termination codon of translation in exon 4. As a consequence, we can predict the rapid degradation of the mutant transcript due to nonsense-mediated messenger RNA decay.30,31 The c.130_147dup18 mutation does not result in a frameshift, but its location within the mitochondrial leader sequence could result in a defective targeting to mitochondria or a deficient processing of the polypeptide. The novel splicing defect IVS4 + 1delG affects the consensus donor splice site of intron 4.32 Unfortunately, RNA from this patient was not available for analysis. Nonetheless, this mutation probably results in exon 4 skipping, as was observed in a previously reported mutation (IVS4 + 1G > C) in this donor site.27

All heterozygous cases presented the low-expression IVS3-48C allele, thereby confirming the common view that clinically overt EPP is associated with inheritance of this allele in trans to a severe FECH mutation.6,12,23,34 The study of the relatives yielded additional confirmation of the importance of this polymorphism in EPP, since all asymptomatic carriers had the IVS3-48T/T genotype. Familial investigation of patient 1 showed that several relatives harbored the A185T mutation, but none had the IVS3-48C allele. Therefore, the phenotype of a hypothetical carrier of the A185T mutation with the low-expression allele in trans remains unknown.

In conclusion, among our series of Spanish patients with EPP, we have found 10 cases with coinheritance of 1 FECH gene mutation with IVS3-48C and 1 rare case with a severe homozygous mutation with very low residual activity. This patient presented a cutaneous photosensitivity similar to other recessive cases but had a higher protoporphyrin accumulation, pronounced microcytic anemia, and early signs of liver involvement. These findings emphasize the need for further research on the genotypic-phenotypic relationships in porphyria.

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REFERENCES