Inheritance in Erythropoietic Protoporphyria: A Common Wild-Type Ferrochelatase Allelic Variant With Low Expression Accounts for Clinical Manifestation

By Laurent Gouya, Herve Puy, Jerome Lamoril, Vasco Da Silva, Bernard Grandchamp, Yves Nordmann, and Jean-Charles Deybach

Erythropoietic protoporphyria (EPP) is a rare autosomal dominant disorder of heme biosynthesis characterized by partial decrease in ferrochelatase (FECH; EC 4.99.1.1) activity with protoporphyrin overproduction and consequent painful skin photosensitivity and rarely liver disease. EPP is normally inherited in an autosomal dominant pattern with low clinical penetrance; the many different mutations that have been identified are restricted to one FECH allele, with the other one being free of any mutations. However, clinical manifestations of dominant EPP cannot be simply a matter of FECH haplinsufficiency, because patients have enzyme levels that are lower than the expected 50%. From RNA analysis in one family with dominant EPP, we recently suggested that clinical expression required coinheritance of a normal FECH allele with low expression and a mutant FECH allele. We now show that (1) coinheritance of a FECH gene defect and a wild-type low-expressed allele is generally involved in the clinical expression of EPP; (2) the low-expressed allelic variant was strongly associated with a partial haplotype [-251G IVS1 – 23T IVS2satA9] that may be ancestral and was present in an estimated 10% of a control group of Caucasian origin; and (3) haplotyping allows the absolute risk of developing the disease to be predicted for those inheriting FECH EPP mutations. EPP may thus be considered as an inherited disorder that does not strictly follow recessive or dominant rules. It may represent a model for phenotype modulation by mild variation in expression of the wild-type allele in autosomal dominant diseases.

SUBJECTS, MATERIALS, AND METHODS

EPP pedigree analysis. Five Caucasian EPP families (Fig 1) were selected on typical clinical and laboratory criteria: the index cases had a classical history of skin photosensitivity and decreased FECH activity in lymphocytes.19 Each parent was characterized either as a transmitter (ie, bearing the deleterious FECH mutation with related decreased FECH enzyme activity) or as a normal parent (ie, with normal FECH enzyme activity). Heterozygosity for at least 1 of the 2 exonic dimorphisms (798G/C and 1520C/T) was a prerequisite for relative FECH mRNA quantitation. Otherwise, ribonuclease protection assay was used in selected cases for absolute quantitation.

Study subjects. (1) Thirty-nine unrelated French Caucasian nuclear EPP families (including the above-described 5 families [77 subjects]) were investigated. The patient group consisted of 39 patients with a documented clinical and laboratory history of EPP. The transmitter parent group consisted of 22 asymptomatic FECH gene carrier parents. The normal parent group consisted of 16 parents with normal FECH...
enzyme activity. (2) The control group consisted of 70 healthy unrelated French Caucasian subjects. The allelic distribution of the 5 polymorphisms satisfied Hardy-Weinberg equilibrium ($\chi^2$ test, $P > .5$). Procedures involving human subjects were performed in accordance with the Helsinki declaration revised in 1983, and informed consent was obtained from all subjects before their inclusion in the study.

Characterization of the specific FECH mutations. The specific FECH gene defect in EPP family 1 has been previously described. In families 2 through 5, the specific FECH gene mutations were characterized by direct sequencing of FECH cDNA and further confirmed at the genomic DNA level. These four mutations were different and not previously described ones (Fig 1). The only missense mutation, the T$^{790}$=C transition in family 4 leading to a serine to proline substitution at position 263 (S263P), was expressed in a prokaryotic system (pGEX-2T vector; Pharmacia Biotech, Uppsala, Sweden) using site-directed mutagenesis (Transformer site-directed mutagenesis kit; Clontech Laboratories, Palo Alto, CA) and showed a residual FECH enzyme activity at 0.5% ± 0.02% (mean ± 2 SD) as compared with 100% for the normal cDNA.

Genotyping for 5 intragenic polymorphisms. Five intragenic polymorphisms distributed over the FECH gene were studied. DNA was prepared from lymphoblastoid cell line as previously described. The IVS1$^{23}$C/T dimorphism was screened as follows. The intron 1/exon 1 junction was amplified by polymerase...
chain reaction (PCR) and then digested by the restriction enzyme Cac8I according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). The −23T allelic fragment is specifically cut by this enzyme. The 798G/C dimorphism in exon 7 was screened as follows. After PCR amplification, exon 7 is digested with Nla III restriction enzyme (New England Biolabs) and the 798C allelic fragment is cut into two parts.

Relative quantitation of FECH mRNA by fluorescent primer extension assay. Total RNA isolated from a lymphoblastoid cell line was reverse transcribed as described.12 Two regions of FECH cDNA were amplified spanning nucleotides +693 to +1273 and +1328 to +1568. Primer extension assay was performed as previously described.12,18,19 Two fluorescent primers were used: 798F, 5'-ITTTTCTGCTCATTCACTG-3'; and 1520F, 5'-CCTTTAATATGTTAA, respectively, located close to polymorphic loci 798G/C (+693/+1273 amplier) and 1520C/T (+1328/+1568 amplier). Extended primers were separated on a 12% DNA automated sequencer (Pharmacia Biotech). Results are expressed as the peak area ratios (R) of the corresponding allelic FECH mRNAs: R798 = (peak area of FECH mRNA bearing a 798G allele)/(peak area of FECH mRNA bearing a 798C allele); and R1520 = (peak area of FECH mRNA bearing a 1520C allele)/(peak area of FECH mRNA bearing a 1520T allele).

Ribonuclease protection assay. In some cases, FECH mRNA was quantified by ribonuclease protection assay as previously described.12 In family 5, the specific gene mutation (IVS2µsatA9→G, T, A9→T) leads to exon 10 skipping, allowing a FECH probe that include exon 10 to be used for quantifying the normal allele independently to the mutated one. Two RNA antisense probes were synthesized: a FECH probe containing the last 414 nucleotides to be used for RNA quantitation. The FECH mRNA was reverse transcribed as described.12 Two regions of a control probe containing the first 125 nucleotides to be used for quantifying the normal allele independently to the mutated one. Two regions of the normal FECH mRNA to the L ferritin mRNA.

Statistical analysis. Genotype and allele distribution was analyzed using the χ² test. Odds ratio and 95% confidence intervals (CI) were calculated.20 The high prevalence of the IVS2µsatA9 allele resulted in a high percentage of homozygotes. The data were then collapsed and analyzed in 2 × 2 format using χ² statistics. Linkage disequilibrium (D) studies were performed according to Thomson et al.21

RESULTS AND DISCUSSION

Five EPP families with dominant inheritance were studied (ie, partial decrease in FECH enzyme activity associated with a specific FECH mutation in one of the FECH alleles in the proband and in one parent; Fig 1). In these 5 families, 5 different FECH mutations were found, and 4 were newly described (Fig 1). Allelic segregation patterns and relative RNA quantitation showed that clinical expression in EPP patients in these families always resulted from the coinheritance of a wild-type low-expressed allele with a mutant one. Haplotype typing using 5 intragenic polymorphisms spread over the 45 kb of the FECH gene from the 5′ promoter region to the 3′ untranslated region (3′ UTR) (−251G/A, IVS1−23CT, IVS2µsat:A:An, 798G/C, and 1520C/T) showed that this low-expression variant was (1) systematically transmitted from the normal parent to the patient and (2) associated with two haplotypes sharing the same 5′ part [G-T-A9] (Fig 1). Not one of these sequence variations was by itself directly involved in the low expression, as attested by allelic segregation in family 1. The normal parent I1,2, although homozygous for the 5′ partial [G-T-A9] haplotype, transmitted a low-expressed allele to the proband (II1,2) but a normally expressed allele to another sibling (II1,1), an asymptomatic carrier of an FECH mutation (Fig 1). Tugores et al22 described many regulatory elements of interest in the promoter region of the FECH gene. Nevertheless, they do not appear to be involved in low-expression mechanism, because no sequence variation has been found in more than 1 kb of the proximal promoter.

In family 5, the specific gene mutation (IVS9-28G-T, Beverly, MA). The invariable presence in the 7 EPP patients (Fig 1) of a bimodal distribution of the quantitation data allowed isolation of a subgroup of 9 subjects with a similar increase in the frequency of a specific set of G-T-A9-T polymorphisms (Table 2). This strongly indicates that the low-expressed allele is associated with a major [G-T-A9-T] haplotype.

The invariable presence in the 7 EPP patients described many regulatory elements of interest in the promoter region of the FECH gene. Nevertheless, they do not appear to be involved in low-expression mechanism, because no sequence variation has been found in more than 1 kb of the proximal promoter.

Recently, Scott et al23 reported a new region 2 kb upstream from the transcription start site that may contribute to a high level of erythropoietic protoxidase expression of FECH gene by maintaining an active chromatin configuration. One might hypothesize that mutations in this region bearing erythropoietic-specific regulatory elements could be involved in the FECH gene low expression. Such a mechanism clearly needs to be evaluated in further studies on FECH gene expression. On the other hand, sequencing the coding regions as well as part of intron 1 and 3′ UTR to search for mutations that might decrease FECH mRNA steady-state level failed to detect any other sequence variations.

These observations based on a limited number of EPP families were extended by a case-control association study in 39 EPP nuclear families in which the distribution of the 5 FECH intragenic polymorphisms was analyzed. The subjects were divided into 3 groups: (1) the patient group, (2) the transmitter parent group (ie, asymptomatic parents bearing a specific FECH mutation), and (3) the normal parent group. The most striking finding was that the patient group and the normal parent group exhibited a similar increase in the frequency of a specific set of alleles (G, T, A9, T; Table 1), one of which has previously been reported to be overrepresented in EPP patients, whereas none of them was associated with the transmitter parent (mutated carrier group). It is thus surprising that such a strong association exists between FECH polymorphisms and overt EPP patients but not with asymptomatic carriers of FECH mutations. This is in agreement with the vast heterogeneity in FECH mutations (38 different ones reported to date). It also assesses that the normal parents transmit to the overt EPP siblings a common but specific wild-type FECH allele. In accordance with their physical proximity, we found evidence for linkage disequilibrium between −251G and IVS1−23T, IVS2µsatA9, and 1520T polymorphisms (Table 2). This strongly indicates that the low-expressed allele is associated with a major [G-T-A9-T] haplotype.

The invariable presence in the 7 EPP patients (Fig 1) of a wild-type low-expressed allele variant with the same 5′ partial [G-T-A9] haplotype implies that this allele should appear with a high frequency in the general population. This was tested in a control group of 70 unrelated normal subjects of whom 39 were heterozygous for one of the exonic dimorphisms 798G/C or 1520C/T and thus available for RNA quantitation. The bimodal distribution of the quantitation data allowed isolation of a subgroup of 9 subjects with FECH allelic mRNA ratios of 0.7 or less. Eight of the nine low-expressed alleles were associated with the same 5′ partial [G-T-A9] haplotype (Fig 2). Haplotype variations for the 3′ polymorphisms indicate that recombination in this part of the gene does not affect expression, suggesting that the mutation(s) causing low expression lies in the 5′ part of an FECH gene with an ancestral [G-T-A9] haplotype. The frequency of the low-expressed FECH allele in the control population could be estimated from 6.5% (9/140, assuming that none of the 31 unexplored subjects had a low-expressed allele variant) to 11.5% (9/78, assuming that this
Table 1. FECH Allele Distribution in 39 Unrelated EPP Families

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Alleles</th>
<th>Controls (n = 70)</th>
<th>EPP Patients (n = 39)</th>
<th>Normal Parents (n = 16)</th>
<th>Transmitter Parents (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−251A/G</td>
<td>G</td>
<td>122</td>
<td>12</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>18</td>
<td>44</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>(49.00, &lt;10^{-4})</td>
<td>(37.29, &lt;10^{-6}) (0.78, NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS1 − 23C/T</td>
<td>C</td>
<td>119</td>
<td>34</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>21</td>
<td>44</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>(41.04, &lt;10^{-4})</td>
<td>(36.7, &lt;10^{-6}) (0.02, NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS2 satAn1-10</td>
<td>1</td>
<td>18</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>0</td>
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<td>0</td>
</tr>
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<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(11.01, &lt;10^{-4})</td>
<td>(3.47, 0.06) (0.69, NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>798G/C</td>
<td>G</td>
<td>104</td>
<td>50</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>36</td>
<td>28</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>(2.5, NS)</td>
<td>(2.8, NS) (0.63, NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1520C/T</td>
<td>C</td>
<td>120</td>
<td>52</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>20</td>
<td>26</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>(10.90, &lt;10^{-4})</td>
<td>(7.11, &lt;10^{-2}) (0.01, NS)</td>
<td></td>
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</table>

Abbreviation: NS, not significant.
*χ² (df = 1), p.
†IVS2 satA9 allele versus other alleles.

Fig 2. Distribution of the FECH allelic mRNA ratios in 39 Caucasian controls. Data are presented as histogram of the observed distribution at 0.1 intervals and a polynomial tendency curve. According to the bimodal distribution, the 39 subjects could be separated into a main subgroup of 30 with equally expressed FECH alleles (mean, 1.02; 95% CI, 0.96 to 1.08) and a smaller group of 9 with a marked disequilibrium in their relative FECH mRNA allelic representation (mean, 0.59; 95% CI, 0.58 to 0.60). Haplotypes for the 9 low-expressed alleles were [G-T-A9-T] in 6 cases, [G-T-A9-C] in 2, and [A-C-A9-T] in 1.

Table 2. Linkage Disequilibrium Between the −251G Allele and Other Polymorphic Loci Within the FECH Gene

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>D*</th>
<th>D†</th>
<th>χ² (df = 1), P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS1 − 23T</td>
<td>0.19</td>
<td>91</td>
<td>36.0, &lt;10^{-3}</td>
</tr>
<tr>
<td>IVS2 satA9</td>
<td>0.10</td>
<td>44</td>
<td>9.7, &lt;5 × 10^{-3}</td>
</tr>
<tr>
<td>1520T</td>
<td>0.07</td>
<td>26</td>
<td>8.9, &lt;2 × 10^{-2}</td>
</tr>
</tbody>
</table>

Performed on 52 haplotypes observed in 26 unrelated Caucasian families.
*Maximum likelihood of linkage disequilibrium. D = h − pq, where p and q are the frequencies of the rare alleles for the two loci analyzed and h is the frequency of the haplotype with the rare alleles at both loci.
†Percentage of D maximum value at the given allele frequencies [Dm = p(1 − q)].
The standardized value of D is Dx = √N √[(p(1 − p)(q(1 − q)))] whose square is asymptotically distributed as a χ² random variable on 1 df. N is the total number of haplotypes used to determine the allelic frequencies of p and q.

absolute quantitation of the nonmutant FECH allele in this family (see Subjects, Materials, and Methods). Compared with the control subject (R = 8.3), FECH mRNA level was 75% lower in the symptomatic father II 5 1 (R = 2.6; 1 deleterious allele and 1 low-expressed one) and 50% lower in the daughter III 5 3 (R = 4.1); as expected, these data were strongly correlated with a similar decrease in FECH enzyme activities (Fig 1). Because no de novo FECH mutation could be found by sequencing (not shown), this gives evidence for the presence in subject III 5 3 of two coinherited low-expressed alleles. This subject has no clinical or biochemical signs of EPP but would have been misdiagnosed as a gene carrier in a family study based solely on FECH enzyme measurement.

These data lead to a number of conclusions. First, that the low expression of a wild-type allelic variant trans to a mutated FECH allele is generally required for clinical expression of EPP. It is likely that when FECH enzyme activity falls below a critical threshold, the accumulation of protoporphyrin will exceed hepatic clearance capacity and lead to clinically manifest EPP. In fact, asymptomatic EPP carriers often have normal or slightly elevated protoporphyrin levels in erythrocytes and higher FECH enzyme activity than overt EPP patients. Second, that the low-expressed alleles are inherited from the normal parent and most if not all of these alleles probably originate from a mutational event(s) that occurred in a common ancestral [G-T-A9] haplotype. However, the mechanism of low expression remains unknown. Third, that the frequency of the low-expressed allele in the general Caucasian population is of the order of 6.5% to 11.5%. Finally, our results have important implications for genetic counseling in EPP. From the frequency of low-expressed alleles bearing specific 5’ haplotypes (−251G IVS1 − 23T or −251A IVS1 − 23C) in the 39 individuals studied, it can be estimated that the absolute risk that a carrier of a FECH mutation will develop symptomatic EPP when bearing the −251G IVS1 − 23T haplotype trans to a mutant one is close to 60%. More importantly, the risk decreases to less than 2% if this haplotype is −251A IVS1 − 23C, which, fortunately, is the
most frequent one (75% of $[-251A \ IVS1-23C]$ homozygotes in the control group). Indeed, in these estimations we assume that the penetrance of EPP symptoms in subjects having a genotype with a mutant $FECH$ allele associated with a low-expressed allele is almost 100% at 15 years of age. As compared with the empirical risk of EPP symptoms proposed by Went and Klasen,24 our results provide a dramatic improvement in risk prediction for an unborn child in an EPP family based on parental haplotype study.

Although autosomal recessive inheritance has been documented in 2 cases,7,8 EPP has long been considered as an autosomal dominant disorder with incomplete penetrance. Molecular analysis supports this notion, because only a single mutation is identified in patients in one of the $FECH$ alleles. However, EPP patients exhibit a lower $FECH$ enzyme activity than the 50% expected in autosomal dominant disease with haploinsufficiency. This strongly suggests a more complex mode of inheritance. Went and Klasen24 proposed a triallelic mode of inheritance. Went and Klasen24 proposed a triallelic

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$$\text{REFERENCES}$$


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