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ORIGINAL ARTICLE

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Novel mutations and phenotypic effect of the splice site modulator IVS3-48C in nine Swedish families with erythropoietic protoporphyria

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Abstract Erythropoietic protoporphyria (EPP) is an inherited disorder, caused by a partial deficiency of ferrochelatase (FECH), the last enzyme of the heme biosynthetic pathway. The deficiency results in accumulation of protoporphyrin, primarily in erythroid cells, and the major clinical feature is cutaneous photosensitivity. In addition, some patients may develop liver complications. Several EPP-coupled mutations have been identified in the *FECH* gene, and the less than 50% of FECH activity seen in patients with overt EPP was recently shown to be due to the *in trans* inheritance of one deleterious mutation and a IVS3-48T>C transition in intron 3 of the *FECH* gene. This IVS3-48T>C transition modulates the use of a constitutive aberrant splice site, which results in a decreased *FECH* mRNA level in the carrier. In the present study, the inheritance of four novel (364C>T, 393delC, 532G>A, and 1088-89insGG) and two previously reported (343C>T and 1001C>T) *FECH* mutations, and the splice site modulator IVS3-48C was investigated in nine Swedish families with EPP. The methods used for the *FECH* gene analysis included denaturating gradient gel electrophoresis, sequencing analysis, and restriction enzyme cleavage. Haplotype analysis, based on the polymorphic loci 287(G/A), IVS3-48(T/C), and 921(G/A), revealed that all individuals carrying a mutated allele and IVS3-48C *in trans* to each other were affected by overt EPP. Mild clinical and biochemical EPP signs may, however, be present in individuals carrying a T at position IVS3-48 *in trans* to a mutated allele, because this was the case in one of the individuals investigated in the present study.

Key words DNA mutational analysis · Heme biosynthesis · Ferrochelatase · *FECH* mutations · Erythropoietic protoporphyria

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Introduction

Erythropoietic protoporphyria (EPP) is a genetic disorder, caused by the inheritance of a partial deficiency of the enzyme ferrochelatase (FECH; EC 4.99.1.1; Bonkowsky et al. 1975). This enzyme, which is the last of the eight enzymes of the heme biosynthetic pathway, catalyzes the insertion of one Fe^{2+} into protoporphyrin IX (Anderson et al. 2001). It operates as a homodimer (Burden et al. 1999) and is located in the mitochondria with the active site facing the matrix (Harbin and Dailey 1985; Jones and Jones 1969). A deficiency of FECH activity results in the accumulation of protoporphyrin, primarily in the erythroid cells of the bone marrow, and the biochemical features of EPP include increased protoporphyrin levels in circulating erythrocytes, plasma, bile, and feces (Anderson et al. 2001). The major clinical characteristic of EPP is cutaneous photosensitivity, which almost always appears in childhood and includes burning, swelling, itching, and redness in sun-exposed areas (Todd 1994). In addition to skin symptoms, hepatic failure occurs in about 1%–10% of EPP patients (Todd 1994), and in some cases liver transplantation has been performed (Bloomer et al. 1998; Polson et al. 1988; Samuel et al. 1988; Thunell et al. 2000).

In symptomatic carriers of EPP traits, the activity of FECH is often only 10%–30% of normal, i.e., less than the 50% that would be expected from an autosomal dominant inherited disease (Todd 1994). A three-allele hypothesis, with a normal allele (F), a deleterious allele (F^{-}), and a “normal” allele with weaker expression (f), was originally proposed by Went and Klasen (1984) after studying the inheritance of EPP in Dutch families. This hypothesis was supported by a study of Gouya et al. (1996) in which the EPP phenotype in an affected family was shown to result from the coinheritance of a low output allele (around 50% lower level) and a mutant allele (exon 10 deletion). The symptomatic patient and asymptomatic carriers of that family exhibited 25% and 50% residual activity, respectively (Gouya et al. 1996). Further investigations of five additional unrelated EPP families carrying different *FECH* mutations

showed that coinheritance of the low expression allele and a mutated *FECH* allele resulted in phenotypic expression of overt EPP and that the low expression allele was strongly associated with the partial 5′-haplotype-251G:IVS1-23T:IVS2μsatA9, which was present in an estimated 6.5%–11.5% of a control group (Gouya et al. 1999). The mechanism responsible for the low expression of FECH was later established to be a IVS3-48T>C transition that modulates the use of a constitutive aberrant acceptor splice site, 63 bp upstream of the normal one (Gouya et al. 2002). The presence of a C at position IVS3-48 was shown to cause 40% aberrantly spliced mRNA, compared with only 20% for the T allele. The reduced level of FECH was due to degradation of the aberrantly spliced mRNA by so-called nonsense-mediated mRNA decay. The C allele was present in 11% of the French control individuals, and the FECH activity in lymphocytes was significantly higher in individuals that were homozygous for T at the IVS3-48 position, compared with individuals that were heterozygous (C/T). Individuals that were homozygous for C showed the lowest FECH activity.

In some cases, EPP is inherited in a recessive manner and a few such homozygous cases (with patients carrying deleterious mutations on both alleles) have been reported (Deybach et al. 1986; Lamoril et al. 1991; Sarkany and Cox 1995).

The cDNA encoding human FECH has been cloned, and the mRNA encodes a precursor enzyme of 423 amino acid residues (Nakahashi et al. 1990), including a mitochondrial targeting sequence spanning amino acid residues 1–54 or 1–62 (Nakahashi et al. 1990; Wu et al. 2001). The gene has been mapped to chromosomal position 18q21.3 (Brenner et al. 1992; Taketani et al. 1992) and contains 11 exons and spans over 45 kb (Taketani et al. 1992). Several EPP-coupled mutations have been identified in the *FECH* gene (Chen et al. 2002; Frank et al. 1999; Gouya et al. 1999; Remenyik et al. 1998; Rufenacht et al. 2001; Yasui et al. 2002; Yotsumoto et al. 2001; Human Gene Mutation Database).

The worldwide prevalence of EPP has been estimated to be 1:75000–1:200000 inhabitants (Todd 1994), and in Sweden this number is at least 1:200000 inhabitants. In 1965 five Swedish families with EPP were reported (Haeger-Aronsen and Krook 1965), and to date there are 26 apparently unrelated families registered at the Porphyria Centre Sweden. In the present study, we investigated the inheritance of mutations and the splice site modulator IVS3-48C in the *FECH* gene in nine Swedish families with EPP.

Subjects and methods

Subjects

Nine EPP probands along with 26 of their relatives were studied. The EPP diagnosis in the probands (and affected relatives) had been based on the appearance of skin symptoms and biochemical signs, including the presence of a fluorescence peak at 635 nm in plasma, elevated erythrocyte porphyrin concentration, and elevated fecal porphyrin concentration. The erythrocyte porphyrin concentrations, shown in Fig. 1, were determined spectrophotometrically (Piomelli 1973). Fifty unrelated, unaffected individuals of Caucasian origin were used as controls. The study was approved by the Ethics Committee of the Karolinska Institute (340/01).

DNA extraction

DNA was extracted from whole blood using QIAamp DNA Mini kit (Qiagen, Chatswell, CA, USA).

Denaturating gradient gel electrophoresis (DGGE)

Denaturating gradient gel electrophoresis (DGGE) was used in the screening for sequence variations in exon 3–11 (along with parts of the flanking introns), in the *FECH* gene of the nine EPP probands. DGGE was also used in the investigation of the polymorphic locus 287(A/G) located in exon 3, and in the subsequent investigation of the mutation status of family members and controls. The primers and conditions that were used are listed in Table 1. The polymerase chain reaction (PCR) mixture used for amplification of fragments suitable for DGGE analysis contained approximately 2 ng/μl genomic DNA, 2 mM $MgCl_2$, 0.15 mM deoxyribonucleoside triphosphates (dNTPs), 2 μM of each primer (Table 1), and 0.025 U/μl Ampli Taq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in Ampli Taq Gold Buffer (Applied Biosystems). The PCR program used was 10 min at 95°C; followed by 40 cycles of 1 min at 95°C; 1 min at 55°C, 58°C, or 60°C (Table 1); and 1 min at 72°C. Thereafter, there was an additional extension for 10 min at 72°C. Samples were then heated to 99°C for 5 min and thereafter incubated for 60 min at 60°C, followed by 60 min at 37°C to allow formation of heteroduplexes. The reaction was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). PCR products, mixed 1:1 with loading dye (Bio-Rad, Hercules, CA, USA), were loaded onto an 8% polyacrylamide gel (acrylamide : bisacrylamide 37.5 : 1) with a linear gradient of denaturant [100% denaturant: 7 M urea and 40% (v/v) formamide; Table 2]. Electrophoresis was performed at 150 V, at 60°C for 5 h, using equipment included in the DCode Universal Mutation Detection System for DGGE (Bio-Rad). For prediction of an appropriate gradient of denaturating agents in the gels, computer analysis of the PCR fragments was performed with Mac Melt software 1.0 (Bio-Rad).

Sequencing analysis

Sequencing analysis of exon 2 (along with parts of the flanking introns) was performed in all nine probands for direct identification of sequence variations, and of exon 3, 4, 5, 9, and 10 (along with parts of flanking introns) to identify the

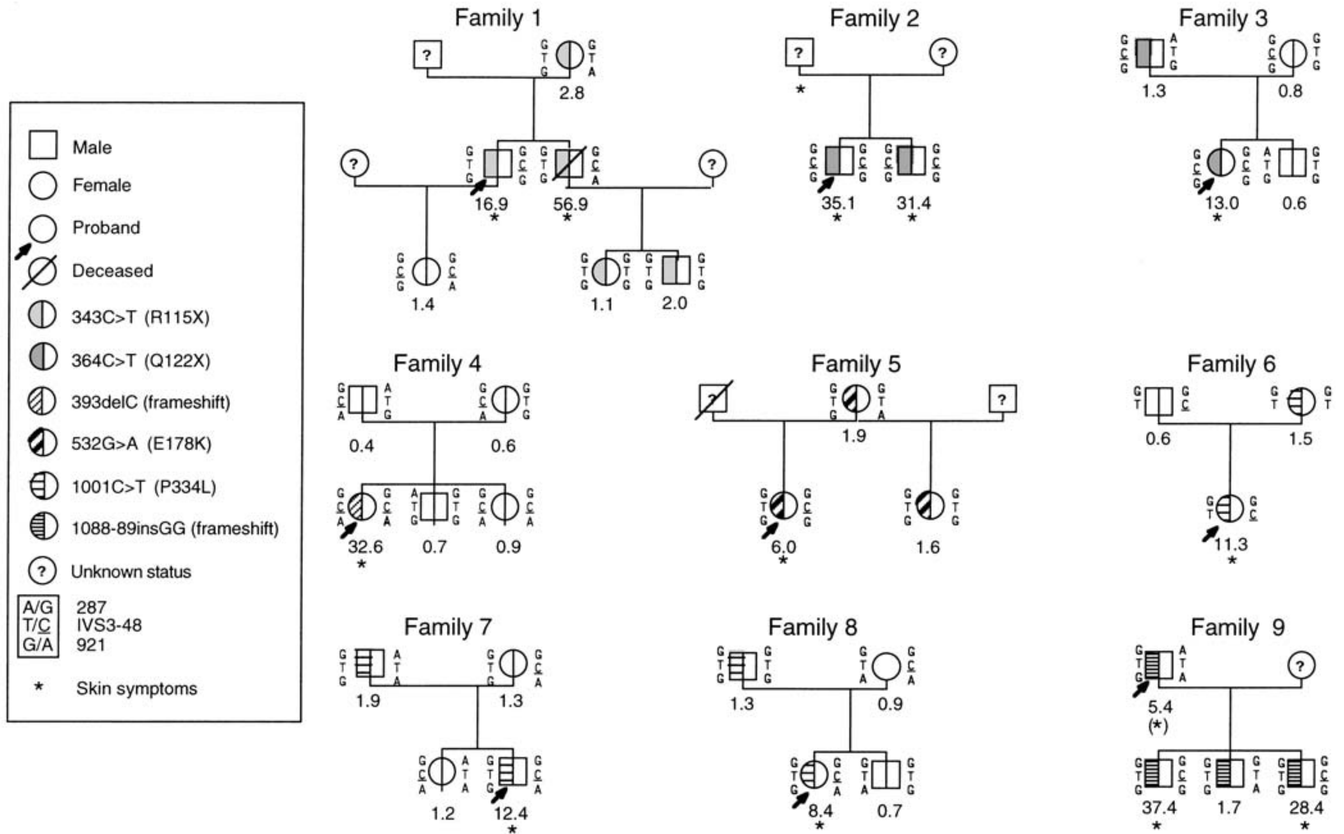

Fig. 1. Pedigrees of Swedish erythropoietic protoporphyria families showing mutation status and haplotypes constructed from the three polymorphic loci, 287, IVS3-48, and 921. Mutation and single-nucleotide polymorphism analyses were performed by denaturating gradient gel electrophoresis or restriction enzyme cleavage. The underlined C (C) at position IVS3-48 denotes the presence of the C nucleotide, which previously has been shown to cause an increased amount of abnormal spliced mRNA, resulting in additional ferrochelatase enzyme deficiency (Gouya et al. 2002). Presented *below* each individual is a representative value of the erythrocyte porphyrin concentration, normal <1.2 μmol/l. The B-hemoglobin values of the individuals were in the normal range at the time for measurement of the erythrocyte porphyrin concentration

Table 1. Oligonuclotide primers and conditions used in the denaturating gradient gel electrophoresis (DGGE) analysis of the ferrochelatase (*FECH*) gene

Exon	Primer	5′-Sequence[a]	Size (bp)	Annealing (°C)	DGGE conditions[b]
3	3S-GC	(GC_{40})-GAAGTGTGACAAATCAACCG	240	60	10%–40%
	3ASb	GTGCCAAGGTTATAATCGAG			
4	4S-GC	(GC_{40})-GCTAAGCTGGAATAAAATCC	276	58	20%–60%
	4ASb	AGAACTAATCTAGTTACATG			
5	5S-GC	(GC_{40})-GGATTAACACTTTCTCC	255	60	20%–50%
	5ASb	GCACCGTATCTAACCTTTCC			
6	6S-GC	(GC_{40})-AATCAAAATGCATCTAC	267	60	10%–60%
	6ASb	CATCCACAAACCCAGAAGGG			
7	7S-GC	(GC_{40})-TTCCCTTCCTCTTTATCCTC	219	60	10%–50%
	7ASb	CCTCTATCACTAGGTCATCC			
8	8S-GC	(GC_{40})-CAGTGTTAATGGCTGAACAC	228	60	30%–60%
	8ASb	CAATAAGAGCTGGCCGCCCG			
9	9Sb	AGGCATAGTCCACTTACGCA	285	60	20%–60%
	9AS-GC	(GC_{40})-ACAAGTCATGATGGGAAAAAGG			
10	10Sb	ATATATCTGTGTTTTTCCTC	180	58	10%–40%
	10AS-GC	(GC_{40})-TCTGGTATGTTCTACTAAACG			
11	11S-GC	(GC_{40})-TGTCCTTCTCATCGGTCTTTG	280	55	30%–60%
	11ASb	TCTCCACATCGGAGGTATCTG			

[a] (GC_{40}) = CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG
[b] Percent denaturant [100% denaturant corresponds to 7 M urea and 40% (v/v) formamide]

Table 2. Oligonucleotide primers used for polymerase chain reaction (PCR) amplification and sequencing of exons 2, 3, 4, 5, 9, and 10 of the ferrochelatase (*FECH*) gene

Exon	Primer	5′-Sequence	Annealing (°C)	Size (bp)
2	2S	ACCTCTTCAGAAGAAATTAC	55	247
	2AS	TATTGGTGTCTGCATCGATA		
3	3S	GTATGGAACAGATTAGAGTTTGC	55	272
	3AS	ACACATGTCAATGTAGTGCC		
4	4S	TGTCAATGACCTCAAGCTTC	55	471
	4AS	AAAGGTCAAGGGATAACGCC		
5	5S	CTTGCCTTCACCGTCAGTGCC	55	419
	5AS	TGGACTGACCTGAACTCTCG		
9	9S	GTAAAGAGTGGCTCTTGCCG	58	526
	9AS	GATGTCAGCCACAGTGATCC		
10	10S	GAGCAGTCTGCGAACAGTTG	55	257
	10AS	CTCAGAGGATTACTCTCTGG		

sequence variations detected by DGGE. DNA fragments were amplified by PCR, using approximately 2 ng/μl genomic DNA, 2 mM $MgCl_2$, 0.15 mM dNTPs, 2 μM of each primer (Table 2), and 0.025 U/μl Ampli Taq Gold DNA Polymerase (Applied Biosystems) in Ampli Taq Gold Buffer (Applied Biosystems). The PCR program was 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C or 58°C (Table 2), and 30 s at 72°C. At the end of the program, there was a final extension for 5 min at 72°C. The reaction was carried out in a Mastercycler Gradient thermal cycler (Eppendorf). The PCR products were purified using QiaQuick PCR Purification kit (Qiagen). The sequencing reaction was performed in either forward or reverse direction using the primers listed in Table 2, together with Big Dye Terminator Cycle Ready Reaction Sequencing kit (Applied Biosystems). The thermal conditions used were 25 cycles of 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C. The samples were analyzed (CyberGene AB, Huddinge, Sweden) on an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Restriction enzyme cleavage

For investigation of the polymorphic locus IVS3-48(T/C) in intron 3, a DNA fragment covering parts of intron 3 and exon 4 was amplified using the PCR conditions as described for sequencing analysis and the primer pair 5′- TGTCA ATGACCTCAAGCTTC-3′ (sense) and 5′-CCTCTCCCT GCTTGGAAGTCC-3′ (antisense). The annealing temperature used in the PCR was 55°C. The 258-bp PCR product was incubated with the restriction enzyme *Tse*I, which cleaves it into two fragments (100 bp + 158 bp) only if a C (and not a T) is present at position IVS3-48.

For identification of the single-nucleotide polymorphism (SNP) 921G>A, which could not be easily discriminated from the 1001C>T transition in the DGGE analysis, exon 9 was amplified by PCR as described for sequencing analysis. The restriction enzyme *Bst*XI cleaves the 526-bp PCR product into two fragments (398 bp + 128 bp) if an A (not G) is present at position 921.

Cleavage was performed for 3 h according to the manufacturer's instructions. The lengths of the resulting fragments were analyzed after electrophoresis on a 3% agarose gel supplemented with ethidium bromide. Both enzymes were from New England Biolabs (Beverly, MA, USA).

Protein sequence alignment

Alignment of protein sequences was performed using Fasta3 version 3.3t09, obtained from The European Bioinformatics Institute (June 5, 2002; http://www.ebi.ac.uk/fasta33).

Numbering system

Exonic nucleotides were numbered from the first nucleotide of the translation initiation codon according to the cDNA sequence derived from the complete *FECH* genomic sequence in GenBank, accession number AJ250235 (http://www.ncbi.nlm.nih.gov/Genbank/). Intronic nucleotides were numbered according to the complete *FECH* genomic sequence, with the exception of the 44 nucleotides immediately upstream of exon 4, which were not present in our material.

Databases

We used the Human Gene Mutation Database (February 5, 2002; http://www.hgmd.org) for reported mutations in the *FECH* gene and the SWISS-PROT database (June 5, 2002; http://www.ebi.ac.uk/swissprot/) for FECH sequences included in the protein sequence alignment.

Results

DGGE analysis, followed by sequencing analysis, identified six different mutations among the nine Swedish EPP probands. Of the identified mutations, 364C>T (exon 4), 393delC (exon 4), 532G>A (exon 5), and 1088-89insGG

(exon 10) are novel, and 343C>T (exon 4) and 1001C>T (exon 9) have been reported previously (Gouya et al. 1998; Henriksson et al. 1996; Rufenacht et al. 1998). None of the mutations were identified among the 50 controls.

The mutation status, haplotype (based on the polymorphic loci 287, IVS3-48 and 921), erythrocyte porphyrin concentrations, and presence or absence of skin symptoms in the 35 investigated individuals are shown in Fig. 1. All carriers of a *FECH* mutation and an IVS3-48C allele *in trans* to each other were shown to have both an elevated erythrocyte porphyrin concentration, ranging between 6.0 μmol/l and 56.9 μmol/l (normal < 1.2 μmol/l), and skin symptoms. Of the individuals carrying a T at position IVS3-48 *in trans* to the mutated allele, only the proband of family 9, who is 68 years old, has experienced (mild) skin symptoms. In this group, the erythrocyte porphyrin concentration is 5.4 μmol/l in the proband of family 9 and ranges between 1.1 μmol/l and 2.8 μmol/l in the individuals without skin symptoms. Two homozygous IVS3-48C/C individuals, not carrying the mutation identified in their respective families, were identified among the investigated families. None of them have experienced skin symptoms and their erythrocyte porphyrin concentrations are 0.9 μmol/l and 1.4 μmol/l, respectively.

The IVS3-48C allele was shown to be associated with two different subhaplotypes, 287G:921G and 287G:921A, in the investigated family members.

The 1001C>T mutation was identified in three of the families (6, 7, and 8), and 364C>T was identified in two families (2 and 3). The remaining mutations were restricted to a single family. In families 7 and 8, the 1001C>T mutation was shown to be associated with haplotype 287G:IVS3-48T:921G. In family 6, this mutation is associated with subhaplotype 287G:IVS3-48T, with either A or G at position 921. In families 2 and 3, the 364C>T mutation is associated with the 287G:IVS3-48C:921G haplotype.

In the 50 controls, the frequencies of the different nucleotides at the three polymorphic loci used for haplotype analysis were 0.17A/0.83G at position 287, 0.92T/0.08C at position IVS3-48, and 0.64G/0.36A at position 921. No individual homozygous for C at position IVS3-48 was identified among these controls.

Discussion

The dominant inheritance of overt EPP has previously been shown to be modulated by a low expression allele (Gouya et al. 1996, 1999, 2002). The underlying mechanism for this low expression involves increased utilization of a cryptic splice site, due to the replacement of T by C at position IVS3-48, resulting in an unstable mRNA transcript (Gouya et al. 2002).

In the present study, four novel and two previously reported mutations were identified among nine Swedish families with EPP. The novel mutations do not seem to represent common SNPs, because none of them were detected among the 50 controls. To investigate the inheritance of mutated alleles and IVS3-48C alleles in the nine families, haplotype analysis based on the polymorphic loci 287(A/G), IVS3-48(T/C), and 921(G/A) was performed.

In EPP family 1, the mutation 343C>T (exon 3) was identified. This mutation has also been identified in a Finnish (Henriksson et al. 1996), a French (Rufenacht et al. 1998), and in a Spanish family (Gouya et al. 1998), and has been shown to correspond to the exchange of arginine for a stop codon (R115X), with a dramatically decreased level of the transcript as a result (Henriksson et al. 1996).

A novel mutation, 364C>T (exon 4), was found in EPP families 2 and 3. On the amino acid level, this mutation corresponds to an exchange of glutamine for a stop codon (Q122X). In both families, the mutation was shown to be associated with haplotype 287G:IVS3-48C:921G, and a common ancestry for these families can therefore not be excluded. In the proband of family 4, the novel mutation 393delC (exon 4) was identified. This mutation is likely to represent a *de novo* mutation, because it was not identified in the asymptomatic parents. The deletion of C causes a frameshift and a premature stop codon. A novel frameshift mutation, 1088-89insGG (exon 10), giving rise to a premature stop, was also identified in family 9. *FECH* mRNA transcribed from an allele containing either 364C>T, 393delC, or 1088-89insGG is likely to be degraded by the nonsense-mediated mRNA decay mechanism (Culbertson 1999), as previously described for 343C>T (R115X) and IVS3-48T>C.

The investigated members of family 5 carry a 532G>A mutation (exon 5), which corresponds to the exchange of the negatively charged glutamate for the positively charged lysine (E178K). The FECH peptide chain has been shown to be folded into two domains, each with a β-α-β motif (Wu et al. 2001), with E178 being located at the end of α-helix number 6. This helix spans amino acid residues 170–179, and has the sequence T-E-E-A-I-E-E-M-E-R. The exchange of a negatively charged amino acid for a positively charged one at this position could possibly affect the H-bonding pattern, resulting in a decreased stability of the protein. Alignment of FECH sequences from different species shows that E178 is conserved among the five species showing the highest degree of overall identity (52.2%–87.9%) to the human FECH sequence (Table 3). In the remaining seven species, however, E178 is not conserved, and in three of the species (*Nicotiana tabacum*, *Cucumis sativus*, and *Hordeum vulgare*) lysine is normally present at this position. This poor conservation of the affected residue indicates that it might not play a very important role in the FECH enzyme. The 532G>A transition was, however, not identified among the 50 controls and can therefore not be considered as a common polymorphism. In addition, in this family, no other putative disease-coupled mutation was identified by the screening method used. Additional mutations might, however, be present in parts of the *FECH* gene that were not investigated, including exon 1, corresponding to a part of the mitochondrial targeting sequence, or other parts of the introns. The disease-coupled mutation could also be one that we were not able to identify by the methods used in the present study, such as a gross deletion. Addi-

Table 3. Alignment of FECH amino acid residues from 13 different species

Species[a]	Overall identity (%)[b]	Amino acid position	
		E178	P334
1. *Homo sapiens* (human)	—	E	P
2. *Mus musculus* (mouse)	87.9	E	P
3. *Bos taurus* (bovine)	87.5	E	P
4. *Gallus gallus* (chicken)	80.8	E	P
5. *Xenopus laevis* (African clawed frog)	79.7	E	P
6. *Drosophila melanogaster* (fruit fly)	52.2	E	P
7. *Saccharomyces cerevisiae* (baker's yeast)	45.5	L	P
8. *Nicotiana tabacum* (common tobacco)	38.1	K	P
9. *Anabaena sp.*	37.7	T	P
10. *Cucumis sativus* (cucumber)	36.1	K	P
11. *Hordeum vulgare* (barley)	34.3	K	P
12. *Deinococcus radiodurans*	31.0	L	A
13. *Bacillus subtilis*	25.3	H	P

Fasta3, version 3.3t09, used for alignment
[a] Accession numbers at SWISS-PROT Database (1) P22830; (2) P22315; (3) P22600; (4) O42479; (5) O577478; (6) Q9V958; (7) P16622; (8) Q949G2; (9) Q8YQR8; (10) P42044; (11) P42045; (12) Q8RV98; (13) P32396
[b] Compared with the normal human sequence

tional investigations are thus needed to elucidate the possible involvement of 532G>A in the pathogenesis of EPP in this family.

Families 6, 7, and 8 were shown to carry the mutation 1001C>T (exon 9), corresponding to the exchange of a conserved proline for a leucine (P334L) (Table 3). This residue is located in the absolute end of β-strand number 7 (spanning the positions 329–334), which is part of the β-sheet in the second domain of the FECH enzyme (Wu et al. 2001). The 1001C>T mutation has previously been identified in a French patient, and in that study this substitution was shown to result in a 19% residual FECH activity (Rufenacht et al. 1998). Because the 1001C>T mutation in families 6, 7, and 8 was shown to be associated with the same haplotype, a common ancestry for these families cannot be excluded.

All carriers of a mutated *FECH* allele and a IVS3-48C allele *in trans* to each other were shown to be affected by overt EPP. The proband of family 9 who carries a 1088-89insGG mutation and is homozygous for T at position IVS3-48 has, however, shown mild skin symptoms in his youth. In addition, his erythrocyte porphyrin concentration is increased (5.4 μmol/l). One of his sons also carries a T at position IVS3-48 *in trans* to the mutation, and, like the other investigated individuals with this genetic constellation, he has only a slightly increased erythrocyte porphyrin concentration (1.7 μmol/l) and no skin symptoms (Fig. 1). The IVS3-48T alleles *in trans* to the mutated allele are associated with different subhaplotypes in the father and his son (287A:921A and 287G:921A, respectively). The 287A>G transition seen in the son corresponds to the exchange Q96R, and has been shown to result in a slight decrease in FECH activity (91.8% of normal) when expressed in *Escherichia coli* (Yasui et al. 2002). The difference at this position is therefore not likely to account for the difference in phenotype between these two individuals. However, there might be additional sequence differences between the two IVS3-48T alleles, causing a decreased level of FECH in the father. In addition, there might be an involvement of other genetic or acquired factors. The two other sons of family 9 also carry the 1088-89insGG mutation, but with a IVS3-48C allele *in trans* to the mutated allele. Both show clear signs of skin manifestation, and their erythrocyte porphyrin concentrations (37.4 μmol/l and 28.4 μmol/l, respectively) highly exceed that seen in their father. The milder or absent skin symptoms and lower levels of erythrocyte porphyrin concentration of their father and brother suggest that also in this family the IVS3-48C allele is involved in modulation of the phenotypic expression of EPP.

It has been suggested that carriers of so-called null-allele mutations in the *FECH* gene are more likely to develop liver disease than are carriers of missense mutations (Chen et al. 2002; Minder et al. 2002). In the present study, null-allele mutations in the form of nonsense and frameshift mutations resulting in premature stop codons were identified in members of family 1 (343C>T, R115X), families 2 and 3 (364C>T, Q122X), family 4 (393delC), and family 9 (1088-89insGG). In family 1, the proband suffered from acute liver failure and died at the age of 50 years 1 month after liver transplantation (Thunell et al. 2000). Liver disease was previously reported in a French patient carrying the 343C>T (R115X) mutation (Rufenacht et al. 1998), but in the Finnish and Spanish patients carrying this mutation no signs of liver disease were reported (Gouya et al. 1998; Henriksson et al. 1996). Of the remaining individuals carrying null-allele mutations in the present study, none have suffered from liver complications so far, nor have carriers of the 532G>A (E178K) or the 1001C>T (P334L) mutation.

In summary, all individuals carrying a mutated *FECH* allele and IVS3-48C *in trans* to each other in the present study were affected by overt EPP. Mild clinical and biochemical EPP signs may, however, be present in individuals

carrying a T at position IVS3-48 *in trans* to a mutated allele because this was the case in one of the individuals investigated in the present study.

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