

Haplotype Analysis in Determination of the Heredity of Erythropoietic Protoporphyrin among Swiss Families

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Defects in the human ferrochelatase gene lead to the hereditary disorder of erythropoietic protoporphyria. The clinical expression of this autosomal dominant disorder requires an allelic combination of a disabled mutant allele and a low-expressed nonmutant allele. Unlike most other erythropoietic protoporphyria populations, mutations identified among Swiss erythropoietic protoporphyria families to date have been relatively homogenous. In this study, genotype analysis was conducted in seven Swiss erythropoietic protoporphyria families, three carrying mutation Q59X, two carrying mutation insT213, and two carrying mutation delTACAG⁵⁸⁰⁻⁵⁸⁴. Three different haplotypes of five known intragenic single nucleotide polymorphisms, namely -251 A/G, IVS1-23C/T, 798 G/C, 921 A/G, and 1520C/T, were identified. Each haplotype was shared by families carrying an identical mutation in the ferrochelatase gene indicating a single mutation event for each of the three mutations. These mutations have been present in the

Swiss erythropoietic protoporphyria population for a relatively long time as no common haplotypes of microsatellite markers flanking the ferrochelatase gene were found, except of two conserved regions, telomeric of the insT213 allele and centromeric of the delTACAG⁵⁸⁰⁻⁵⁸⁴ allele, each with a size > 3 cM. Among the nonmutant ferrochelatase alleles, patients from six erythropoietic protoporphyria families shared a common haplotype [-251G; IVS1-23T] of the first two single nucleotide polymorphisms. An exception was the haplotype [-251 A; IVS1-23C] identified in the index patient of one erythropoietic protoporphyria family. These results supported the recent findings that the low expressed allele is tightly linked to a haplotype [-251G; IVS1-23T] of two intragenic single nucleotide polymorphisms in the ferrochelatase gene. Key words: ferrochelatase/founder effect/intragenic SNP/recurrent mutation. *J Invest Dermatol* 117:1521-1525, 2001

Erythropoietic protoporphyria (EPP, MIM 177000) is a genetic disorder of porphyrin metabolism caused by a partial deficiency of ferrochelatase activity. Ferrochelatase (FECH; EC 4.99.1.1), the last enzyme of the heme biosynthetic pathway, catalyzes the insertion of Fe²⁺ ion into protoporphyrin IX to form heme (Kappas *et al*, 1995). Defective ferrochelatase leads to the accumulation of protoporphyrin IX, and occurs mainly in the red blood cells. Clinically, an excess amount of free protoporphyrin deposited in the skin causes an extremely painful photosensitivity in EPP patients that starts in early childhood. In addition to cutaneous photosensitivity, a small percentage (<2%) of patients develop hepatobiliary complication as a result of the toxic effect of protoporphyrin on the liver (Bloomer *et al*, 1998).

The human FECH gene localized on chromosome 18q contains 11 coding exons and has a size of approximate 45 kb; the entire cDNA as well as partial genomic sequences have been published

(Nakahashi *et al*, 1990; Whitcombe *et al*, 1991; Taketani *et al*, 1992). Mutations in the human FECH gene have been shown to be associated with the decrease in ferrochelatase activity in EPP. To date, over 60 different FECH gene mutations have been published in EPP patients from various countries and ethnic backgrounds (Schneider-Yin *et al*, 2000a). The majority of the mutations are family specific with a few exceptions of mutations being shared by a limited number of EPP families. In Ireland, a single base pair deletion (delG40) was identified in three EPP families (Todd *et al*, 1993). In the U.S.A, mutations K379X and IVS1 + 5, g→a (del exon 1), were each shared by four EPP families (Wang *et al*, 1999). In Switzerland, recurrence of FECH gene mutations seems to be a rather frequent phenomenon. In the past, we have identified a total of five different mutations in the FECH gene of 13 Swiss EPP families. Four of them, namely mutations Q59X, insT213, delTACAG⁵⁸⁰⁻⁵⁸⁴, and del TG⁸⁹⁹⁻⁹⁰⁰, were found in more than one apparently unrelated family (Rüfenacht *et al*, 1998a). Interestingly, mutation delTACAG⁵⁸⁰⁻⁵⁸⁴ has also been detected in EPP patients from France and the U.S.A. (Bloomer *et al*, 1998; Rüfenacht *et al*, 1998a).

In general, molecular defects underlining EPP are heterogeneous, although mutations identified among patients with liver complications share a common feature of producing a truncated enzyme. These so-called "null allele" mutations consist of frame-shifts and nonsense mutations. The association between null allele

Manuscript received May 29, 2001; revised August 21, 2001; accepted for publication September 18, 2001.

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; FECH, ferrochelatase; SNP, single nucleotide polymorphism.

Table I. Haplotypes of the mutant FECH alleles among patients and asymptomatic carriers from EPP families

Locus ^a	Family	Family						
		Q59X			ins T ²¹³		delTACAG ⁵⁸⁰⁻⁵⁸⁴	
		I	II	III	IV	V	VI	VII
D18S69	(0.78; 60.67 cM)	3	4	2	3	3	2	2
D18S1152	(0.80; 62.20 cM)	5	5	4	4	1,4	4	4
D18S41	(0.77; 62.76 cM)	2	2	2	4	2	2	2
D18S858	(0.77; 62.91 cM)	5	4	5	5	3	6	6
-251 A/G	(promoter)	G	G	G	A	A	A	A
IVS1-23 C/T	(intron 1)	T	T	T	C	C	C	C
798 G/C	(exon 7)	C	C	CG	G	G	C	C
921 G/A	(exon 9)	GA	G	G	G	G	A	A
1520 C/T	(3'-UTR)	C	C	C	C	C	C	C
D18S381	(0.62; 63.80 cM)	6	6	4	4	4	3	2
D18S977	(0.91; 64.93 cM)	2,6	6	6	1	1	6	6
D18S1144	(0.85; 65.27 cM)	8	8	7,12	10	10	10	7
D18S1155	(0.67; 65.58 cM)	1	1,4	3	4	4	4	4
D18S64	(0.74; 67.28 cM)	5	3	4	4	3,4	5	5

^aMarker order, heterozygosity, and marker distance were obtained from both LDB and GDB (see *Materials and Methods*). Heterozygosity and map position (cM) are given in parentheses. The FECH gene is located at 63.76 cM.

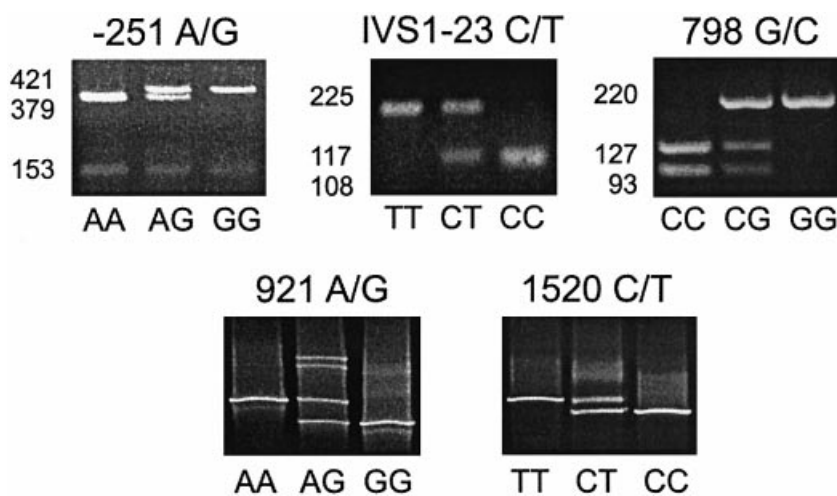


Figure 1. Analyses of five intragenic SNP in the FECH gene. SNP -51 A/G in the promoter region, -23C/T in intron 1, and 798 G/C in exon 7 were analyzed by PCR amplification of genomic DNA and subsequent restriction enzyme analyses using *A*luI, *C*ac81, and *N*laIII, respectively. SNP 921 A/G in exon 9 and 1250 C/T in the 3'-UTR were analyzed by PCR amplification of genomic DNA, followed by DGGE analysis.

mutations and liver complications in EPP is statistically significant, $p < 0.05$ (Schneider-Yin *et al*, 2000a).

The heredity of EPP has a complex pattern and cannot be simply defined as either autosomal dominant or autosomal recessive inheritance. In the majority of EPP families, a single mutation is the only identifiable molecular defect in the FECH gene. Among all carriers of a FECH gene mutation, less than 10% of the individuals will develop the clinical symptoms of EPP, the majority remaining asymptomatic throughout their lives. Various studies have shown that, at the level of enzyme activity, symptomatic patients will exhibit a value 10%–30% of normal, whereas asymptomatic carriers will have approximately 50% of normal enzyme activity (Deybach *et al*, 1986; Norris *et al*, 1990). The recent work by Gouya and colleagues has led to the disclosure of the low expression mechanism – a step forward towards the understanding of the low clinical penetrance in EPP (Gouya *et al*, 1996; 1999). Among the patients of EPP families they studied, those with lower than 50% of normal ferrochelatase activity resulted from a reduced mRNA transcription of the nonmutant FECH allele. Two single nucleotide polymorphisms, -251 A/G in the promoter region and IVS1-23C/T, have been shown to be tightly linked to the low expressed FECH allele (Gouya *et al*, 1999).

To explore the unique feature of recurrent FECH gene mutations in the Swiss EPP population, haplotype analyses using both intragenic single nucleotide polymorphisms (SNP) and microsatellite markers were conducted among seven EPP families carrying three frequent mutations. The data obtained from this study indicated common ancestral alleles for each of the three mutations, Q59X, insT213, and delTACAG⁵⁸⁰⁻⁵⁸⁴. In addition, the role of two intragenic SNP (-251 A/G and IVS1-23C/T) on the clinical penetrance of EPP was examined in this EPP cohort.

MATERIALS AND METHODS

Patients and specimens A total of 31 individuals from seven unrelated Swiss EPP families, including 11 patients, 11 asymptomatic mutation carriers, and nine healthy individuals, were studied. Peripheral blood samples were collected from these individuals with informed consent. The study design was approved by the ethical committee of Stadtpital Triemli, Zürich.

Mutations in the FECH gene Among the seven EPP families, three families (I, II, and III) carried mutation Q59X, two families (IV and V) carried mutation insT213, and two families (VI and VII) carried mutation delTACAG⁵⁸⁰⁻⁵⁸⁴ in the FECH gene, as was previously published (Schneider-Yin *et al*, 1994, 1995; Rüfenacht *et al*, 1998a, b).

Table III. Distribution of GT and AC haplotypes among the nonmutant FECH allele of EPP patients and asymptomatic mutation carriers

Subjects	Haplotypes	
	[-251 G; IVS1-23T]	[-251A; IVS1-23C]
Patients (<i>n</i> = 11)	10	1
Carriers (<i>n</i> = 11)	0	11
Controls (<i>n</i> = 64) ^a	13/13	51/51

^aAmong 32 control samples, analysis of both FECH alleles resulted in the identification of an equal number (13) of Gs at -251 and Ts at IVS1-23, and an equal number (51) of As at -251 and Cs at IVS1-23.

Intragenic SNP in the FECH gene Five known intragenic SNP in the human FECH gene, namely -251 A/G in the promoter region, IVS1-23C/T, 798 G/C in exon 7, 921 A/G in exon 9, and 1520C/T in the 3'-UTR, were analyzed in all members of the seven EPP families. In addition, -251 A/G and IVS1-23C/T were determined in 32 control samples.

Genomic DNA was isolated from peripheral blood of all subjects using a QIAamp Blood kit (Qiagen, Germany). To analyze -251 A/G, the promoter region of the FECH gene was amplified by using a sense primer 5'-CCG TCC CTC CAA GAA ATG and an antisense primer 5'-GGT GTC CGC CCA GCA GTG. The 574 bp product was digested with restriction enzyme *Alu* I (Boehringer Mannheim, Germany). Partial sequence of intron 1 was amplified by using a sense primer 5'-TTA CCT GCC TGC AGA GAA ATC A and an antisense primer 5'-GCT GGG CTG TTT CTG TGG TG for determination of IVS1-23C/T. The 235 bp product was digested with restriction enzyme *Cac* I (New England Biolabs, U.K.). Conditions for polymerase chain reaction (PCR) amplifications of exon 7 and 9 of the FECH gene and the subsequent denaturing gradient gel electrophoresis (DGGE) analysis of exon 9 have been previously described (Rüfenacht *et al.*, 1998a). Following the PCR, the 220 bp product of exon 7 was digested with restriction enzyme *Nla* III (New England Biolabs). All enzyme digests were analyzed in a 3% agarose gel. The 3'-UTR of the FECH gene was amplified by PCR using primers as described by Gouya *et al.* (1996). The PCR product was analyzed by DGGE on a D GENE System (Bio-Rad, Hercules, CA) with a linear denaturant gradient of 13%–43%. The DGGE gel (16 × 16 cm) was run at 60°C for 3 h under a constant voltage of 120 V.

Microsatellite markers flanking the FECH gene In addition to the intragenic SNP, a total of nine microsatellite markers were selected for haplotype analysis. Most of the microsatellites were chosen from the Marshfield map (Broman *et al.*, 1998) for maximum informativity. These markers and additional polymorphisms are located in the genomic region of the FECH gene in the order of cen-D18S69-D18S1152-D18S41-D18S858-FECH-D18S381-D18S77-D18S1144-D18S1155-D18S64-qter in the LDB map (Collins *et al.*, 1996). The marker heterozygosities and marker distances are given in Table I. PCR primers were either purchased (Research Genetics, Huntsville, AL) or synthesized with the available sequence information. PCR was performed using standard conditions. After a 5 min heat denaturation at 95°C, the PCR products were separated on 4%–6% (depending on the size of the alleles) polyacrylamide sequencing gels of 17 × 50 cm (BioRad, Hercules, CA) under a constant power of 50 W for 1.5–2.5 h (depending on the size of the alleles). DNA fragments were visualized by silver staining (Haider *et al.*, 2000). Arbitrary numbers were assigned to different alleles in the analyzed population, beginning with the largest allele.

RESULTS

Characterization of intragenic SNP Allelic characterization of five SNP in the FECH gene is shown in Fig 1. A 574 bp DNA fragment in the promoter region bearing the A/G dimorphism at position -251 was amplified by PCR. The presence of A or G at -251 position was verified by *Alu* I digestion of the PCR product. Homozygous A/A appeared as three bands of 379 bp, 153 bp, and 42 bp, whereas homozygous G/G appeared as two bands (421 and 153 bp) on the agarose gel. The C/T dimorphism at position -23 near the intron 1–exon 2 junction was characterized by *Cac* I

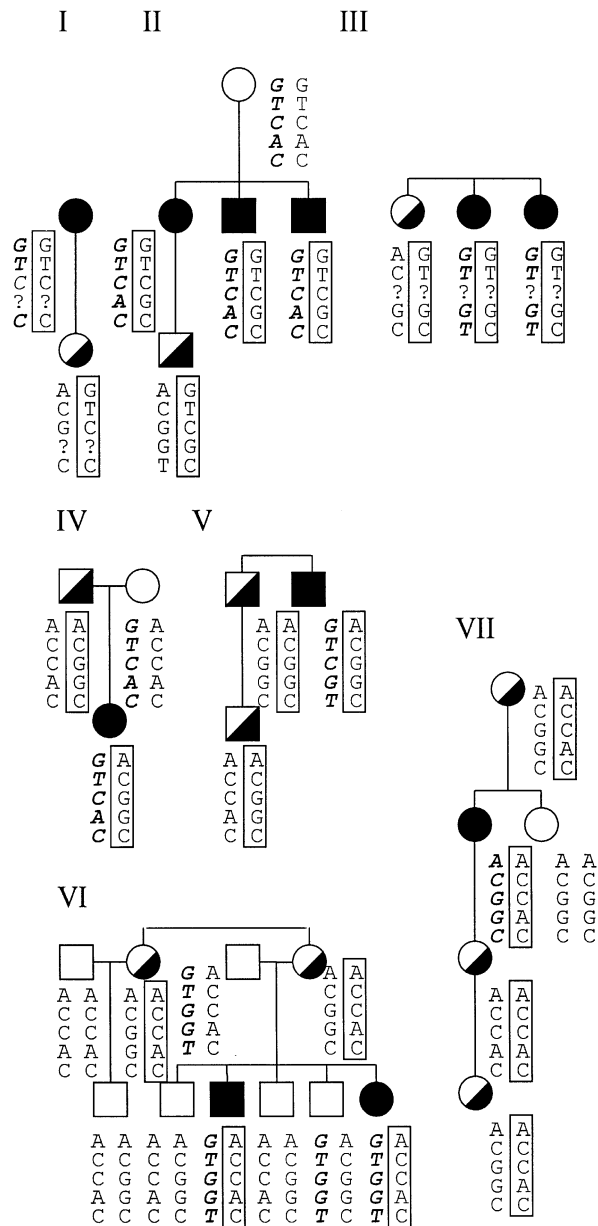


Figure 2. Haplotype analysis among Swiss EPP families using intragenic SNP of the FECH gene. Patients, asymptomatic carriers, and normal individuals are indicated by solid, half-filled, and empty symbols, respectively. The FECH gene alleles carrying a disabling mutation are drawn in a rectangular frame. The alleles that are responsible for the disease onset are in bold and italic. The SNP positions at which all available members of a pedigree are heterozygous are indicated by a question mark.

digestion of PCR-amplified genomic DNA. The PCR product was cut into three fragments of 117 bp, 108 bp, and 10 bp in the case of homozygous C/C, and was cut into two fragments of 225 bp and 10 bp in the case of homozygous T/T. A 220 bp fragment spanning over exon 7 of the FECH gene was subjected to *Nla* III digestion to determine the status of 798 G/C dimorphism. The 220 bp fragment remained intact if a G was present on both alleles. The presence of C at position 798 introduced a *Nla* III site. The PCR product from a C/C homozygote was therefore digested into two fragments of 127 bp and 93 bp by the enzyme.

SNP 921 A/G and 1520 C/T, located in exon 9 and the 3'-UTR of the FECH gene, respectively, were characterized by DGGE analysis. As shown in Fig 1, both 921 G/G and A/A

Table II. Haplotypes of the nonmutant FECH alleles among patients of EPP families

Locus	Family						
	I	II	IV	III	V	VI	VII
D18S69	4	3	6	3	3	6	5
D18S1152	5	5	3	6	1,4	3	3
D18S41	4	2	3	4	2	4	2
D18S858	5	4	5	5	3	3	4
-251 A/G	G	G	G	G	G	G	A
IVS1-23 C/T	T	T	T	T	T	T	C
798 G/C	C	C	C	CG	C	G	G
921 G/A	AG	A	A	G	G	G	G
1520 C/T	C	C	C	T	T	T	C
D18S381	6	6	6	4	4	6	2
D18S977	2,6	2	4	2	2	3	2
D18S1144	12	10	10	7,12	5	12	3
D18S1155	1	1,4	4	3	3	1	1
D18S64	2	5	5	3	3,4	5	4

homozygotes appeared as a single band, but with different migration rates in the DGGE gel. The A/G heterozygote showed a characteristic pattern of quadruple bands in the gel. In the case of the 1520 C/T dimorphism, the C/T heterozygote exhibited a pattern of double bands, whereas the single bands from both C/C and T/T homozygotes differed by their migration rates in the DGGE gel.

Haplotype analysis using intragenic SNP Mutations in the FECH gene of symptomatic patients among seven EPP families were identified by previous sequencing analysis (Schneider-Yin *et al*, 1994, 1995; Rüfenacht *et al*, 1998a). Other family members were screened for the family-specific mutation by either DGGE or restriction analyses to identify asymptomatic mutation carriers (Rüfenacht *et al*, 1998a, b). The statuses of patient, carrier, and unaffected family member are indicated in the pedigrees in **Fig 2**. Based on this information, haplotypes featured by five intragenic SNP were defined in five pedigrees according to Mendelian inheritance. In families I and III, however, 798 G/C could not be aligned with other markers because all tested individuals from these families were heterozygous G/C.

As presented in **Fig 2**, among all 62 alleles from 31 tested individuals, including patients, carriers, and healthy relatives of the seven EPP families, -251 A was found invariably *in cis* to IVS1-23C, and -251G was at all times *in cis* to IVS1-23T.

The mutant FECH allele Haplotypes of the mutated FECH alleles from the seven EPP families carrying three different FECH mutations are illustrated separately in **Table I**. Each of the three mutations was found to cosegregate with one particular haplotype of the five intragenic SNP in all families carrying the respective mutations. Families I, II, and III, carrying the Q59X mutation, shared a common haplotype of [G-T-C-G-C]. Families IV and V, both carrying the insT213 mutation, shared a common haplotype of [A-C-G-G-C]. Families VI and VII, both carrying the delTACAG⁵⁸⁰⁻⁵⁸⁴ mutation, shared a common haplotype of [A-C-C-A-C].

The nonmutant FECH allele As, as stated above, the nonmutant FECH allele determines the clinical outcome of EPP, haplotypes of patients were compared with those of the asymptomatic carriers. As shown in both **Fig 2** and **Table II**, the nonmutant FECH allele in 10 EPP patients from six families exhibited a common haplotype of [-251G; IVS1-23T] at the first two SNP. Only one patient in family V showed a haplotype of [-251A; IVS1-23C] in the nonmutant FECH allele. In contrast, the nonmutant FECH allele of all 11 asymptomatic carriers in this study cohort featured the haplotype [-251A; IVS1-23C] (**Fig 2, Table III**).

Haplotype analysis using microsatellite markers

The mutant FECH allele As described above, genotype analysis using intragenic SNP indicated identical haplotypes for the three most common FECH gene mutations in Switzerland. To further investigate this phenomenon, nine microsatellite markers were analyzed in all members of the seven EPP families. The microsatellites flank the FECH gene on chromosome 18q22 and span ≈6.6 cM. In general, the results showed that no extended conserved haplotypes were associated with the FECH gene mutations in the analyzed genomic areas except for two regions. As shown in **Table I**, one conserved telomeric region of the insT213 allele spans ≈3.5 cM; the other conserved centromeric region of the delTACAG⁵⁸⁰⁻⁵⁸⁴ allele spans ≈3.1 cM.

The nonmutant FECH allele Various haplotypes in the extended genomic regions were observed among the seven nonmutant alleles from EPP patients (**Table II**). In particular, no apparent similarities were found among the six microsatellite haplotypes (from families I to VI) that were linked to the common intragenic SNP haplotype of [-251G; IVS1-23T].

DISCUSSION

Based on the available genetic information, mutations in the human FECH gene are typically heterogeneous or family specific. Among the few exceptions, mutations in the Swiss EPP families have been found to be relatively homogenous (Schneider-Yin *et al*, 1994, 1995; Rüfenacht *et al*, 1998a, b). In this study, we carried out genotype analysis in order to test the hypothesis that families carrying an identical FECH mutation are derived from a common ancestor. This hypothesis was verified insofar as an intragenic SNP haplotype was conserved among analyzed patients, providing evidence for a single mutation event for each of the three FECH gene mutations in the Swiss population. In addition, two conserved areas, telomeric of the insT213 allele and centromeric of the delTACAG⁵⁸⁰⁻⁵⁸⁴ allele, were identified by analyses of extragenic polymorphisms covering a genomic region of 6.6 cM flanking the FECH gene. No conserved microsatellite haplotypes were found to be associated with any of the three mutations, however. Although these results do not allow an accurate calculation of the age of the mutations, it is reasonable to conclude that these recurrent FECH gene mutations have been present in the Swiss EPP population for a relatively long time.

Recurrent mutations or "founder effects" have been observed in other disease-associated populations in Switzerland. Molecular studies of the porphobilinogen deaminase gene unveiled a common mutation of W283X, with a prevalence of around 60%, among Swiss patients with acute intermittent porphyria (Schneider-Yin *et al*, 2000b; Schuurmans *et al*, in press). A further example for a recent founder effect in the Swiss population is the high incidence (approximately 5%) of the cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation insT3905 among patients with cystic fibrosis, as evidenced by its association with a conserved haplotype of three highly polymorphic intragenic microsatellites (Hergersberg *et al*, 1997). It is therefore tempting to speculate that the Swiss population is at least partly constituted from relatively homogeneous subpopulations.

As shown in this study, the utilization of intragenic SNP in the FECH gene played a decisive role in the search for ancestral founders of various mutations. Our results supported the view that SNP, due to their low mutation rates, are appropriate markers for the identification of founder haplotypes. Using extragenic microsatellite markers, Wang and colleagues studied haplotypes of EPP patients from unrelated families carrying identical FECH gene mutations commonly found in the U.S.A. (Wang *et al*, 1999). The questions raised from their study as to whether the two common mutations were "hotspots" or "dispersed ancestral mutant alleles" in the FECH gene might be clarified by the analysis of the intragenic SNP haplotypes.

The hereditary mode of EPP has long been a discussion point. The description "an autosomal dominant disorder with a low

clinical penetrance" has been the most commonly used term in EPP, although in some respects the disease resembles an autosomal recessive disorder. Based on clinical and biochemical analyses of over 200 EPP patients, Went and Klagen proposed a "third allele" hypothesis to explain the low clinical penetrance in EPP (Went and Klagen, 1984). In this hypothesis, an unknown factor or a "third allele" in addition to the defective enzyme was postulated to be responsible for the clinical manifestation of EPP. The hypothetical "third allele" was substantiated through the identification of a low expressed FECH allele in a number of EPP families (Gouya *et al*, 1996, 1999). Hence, the full expression of EPP phenotype requires the coinheritance of a "normal" FECH allele that has low expression and a mutant FECH allele (Gouya *et al*, 1999).

The extensive genetic information gathered in this study enabled us to verify Gouya's latest findings. As seen in all pedigrees, a disease-precipitating nonmutant allele cosegregated with the mutant FECH allele in patients. These nonmutant, presumably low expressed, alleles were characterized by an SNP haplotype [-251G; IVS1-23T] in six out of seven families. With the exception of the two SNP, no other common features, either intragenic or extragenic, were observed among the nonmutant alleles in all seven EPP pedigrees.

Undoubtedly, the [-251G; IVS1-23T] haplotype is strongly linked to the low expressed FECH allele, as Gouya *et al* concluded in their studies, as the same haplotype was identified in the low expressed normal FECH alleles from the six French EPP families tested (Gouya *et al*, 1996, 1999). We did find an exception of a nonmutant FECH allele carrying [-251A; IVS1-23C] in one pedigree (family VII), however. Interestingly, although the index patient in this family suffers from typical clinical symptoms of EPP, the mother of the index patient, now aged 74, used to be symptomatic until the age of 35.

Except for the fact that the low expressed FECH allele has a reduced mRNA output, little is known about the mechanism of low expression. The role of SNP -251 A/G on the transcriptional activity of FECH was recently examined by Magness *et al* (2000). Two FECH promoters of 1.1 kb, one with an A and the other with a G at position -251, were cloned separately in front of a reporter gene. No differences in terms of the amount of protein generated by the reporter gene were observed between the two promoters. The C→T transition at position -23 in intron 1 can indeed cause the skipping of exon 2 as observed by Nakahashi *et al* (1992). IVS1-23T, however, by way of exon 2 skipping, is certainly not the only explanation for the low mRNA output as, at least in family VII, the EPP patient was homozygous C/C at IVS1-23. The continuing search for a functional mutation in the low expressed FECH allele may eventually lead to the clarification of the low expression mechanism in EPP.

The authors wish to thank Dr. David Betts for his help in the preparation of this manuscript. This work was supported by the Swiss National Science Foundation, grant 31-53799.98.

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