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THE MOLECULAR GENETICS OF ERYTHROPOIETIC PROTOPORPHYRIA

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Abstract – Erythropoietic protoporphyria (EPP) is a syndrome in which accumulation of protoporphyrin IX in erythroid cells, plasma, skin and liver leads to acute photosensitivity and, in about 2% of patients, liver disease. More than 95% of unrelated patients have ferrochelatase (FECH) deficiency (MIM 177000) while about 2% have X-linked dominant protoporphyria (XLDPP) (MIM 300752) caused by gain-of-function mutations in the *ALAS2* gene. Most FECH-deficient patients are compound heterozygotes for a hypomorphic allele (*FECH* IVS3-48C) and a deleterious *FECH* mutation that together lower FECH activity to around 30% of normal. The frequency of the IVS3-48C allele varies between populations, ranging from less than 1% to 45%. About 4% of unrelated FECH-deficient patients are compound heterozygotes or homozygotes for rare *FECH* mutations and have lower enzyme activities. Acquired somatic mutation of *FECH* secondary to myeloid disease may rarely cause EPP. The risk of liver disease is increased in XLDPP and in FECH-deficient patients who are hetero- or homoallelic for rare *FECH* mutations. Inherited FECH-deficient EPP is an autosomal recessive disorder with some families showing pseudodominant inheritance; the proportion of such families being determined by the population frequency of the IVS3-48C allele.

Key words: Protoporphyria, molecular genetics, ferrochelatase, 5-aminolevulinate synthase, mutation

INTRODUCTION

Erythropoietic protoporphyria (EPP) (MIM 177000) is a syndrome in which life-long acute photosensitivity results from excess protoporphyrin IX in erythroid cells, plasma and skin (6,11,40). Protoporphyrin also accumulates in the liver leading to severe liver disease in about 2% of patients (6,20,40). Mild microcytic anaemia and thrombocytopenia are common and may indicate a direct effect of excess protoporphyrin on haematopoiesis (13,18). In most patients, accumulation of protoporphyrin results from partial deficiency of ferrochelatase (FECH; EC 4.99.1.1) (4,8) which catalyses the chelation of ferrous iron by protoporphyrin IX. Symptoms do not normally occur unless FECH activity is decreased to at least 35% of normal (4,8,25). In a few patients, FECH activity is normal and protoporphyria results from increased activity of 5-aminolevulinate synthase 2 (ALAS2), the rate controlling enzyme of erythroid haem synthesis (43).

With only rare exceptions (7), EPP is an inherited syndrome. Even after the identification of mutations in the *FECH* gene (15,32) in EPP, the precise pattern of inheritance remained uncertain. The difference in FECH activity between clinically overt and latent individuals (25) could not readily be explained by the prevailing view that inheritance was autosomal dominant with low clinical penetrance (30) but was consistent with a three allele system (26,42). Research during the past decade has identified three separate patterns of inheritance of EPP. Here we review current knowledge of the molecular genetics of this disorder.

THE HUMAN *FECH* GENE

Human FECH is a homodimeric, 86kDa protein associated with the mitochondrial inner membrane (48). It is encoded by a single gene at chromosome 18q21.31 that contains 11 exons spread over 45kb (39). A single transcript is translated to produce an identical functional protein in all tissues. The *FECH* promoter contains erythroid-specific and HIF-1 elements that direct its enhanced expression during erythroid differentiation and in response to hypoxia (17,41). A pseudogene has been identified at chromosome 3p22-p23 (46).

Three synonymous and six non-synonymous SNPs, together with a large number of intron SNPs and other sequence variants have been identified in human *FECH* (www.ncbi.nlm.nih.gov/snp/). Identification and sequencing of haplotypes segregating with EPP lead to the discovery of a dimorphism in intron 3 (*FECH* IVS3-48C/T); one allele (IVS3-48C) of which has a FECH activity about 15-20% lower than the other (Table 1)(8,9). The decrease in activity is caused by enhanced use of a cryptic acceptor slice site that produces a truncated, unstable mRNA (9). This dimorphism is an important determinant of FECH activity but has little effect on erythrocyte porphyrin concentrations in normal individuals (Table 1). The frequency of the IVS3-48C allele varies remarkably between populations (Table 2), showing a north-south and east-west gradient that ranges from less than 1% in West Africa to 45% in Japan, and has a positive correlation with the prevalence of EPP (8). The phylogenic origin of the IVS3-48C haplotypes strongly suggests that the IVS3-48C allele originates from a single mutational event (9). At present, it is unclear whether genetic drift alone or positive selection explains its rapid expansion across Asia. There is no evidence for other functional SNPs in *FECH*.

MOLECULAR ANALYSIS IDENTIFIES DIFFERENT PATTERNS OF INHERITANCE IN EPP

FECH mutation on one allele

In most patients with EPP, a *FECH* mutation that markedly decreases or abolishes enzyme activity can be identified on only one allele (1,8,10,31,32,35,47). About 90% of UK families are in this category. The discovery that clinical expression of this type of EPP normally required a hypomorphic IVS3-48C allele *trans* to the mutation (9), was independently confirmed by studies from Japan, North America, Sweden, Israel, South Africa and the United Kingdom (24,28,31,37,45,47). Among 259 patients with a single *FECH* mutation from France and the UK, only 4 (1.5%) have the IVS3-48T/T genotype.

Lymphocyte FECH activities are decreased by 70% (mean ± SD: 1.45 ± 0.32 nmol zinc-mesoporphyrin/h/mg; n = 90) in this group of patients; a decrease that is consistent with the combined effects of a deleterious *FECH* mutation and a hypomorphic allele. The same mutations *trans* to a IVS3-48T allele decrease activity by 52% (2.42 ± 0.41 nmol/h/mg; n = 61). This marked decrease suggests that the mutations in this type of EPP markedly decrease or abolish FECH activity. Molecular studies have revealed allelic heterogeneity (8,31,32) though large extended families account for 80% of South African patients of European descent and two mutations account for 57% of Swiss families(28,36). Less than half the mutations are missense; the remainder are nonsense, frameshifts, large deletions or affect RNA splicing (8,44). Prokaryotic or eukaryotic expression of these missense mutations has generally shown FECH to be less than 10% of control activities; a notable exception being P334L which has an activity of 19% of normal (32). Patients with this mutation have lower erythrocyte protoporphyrin concentrations (median 11.1 μmol/L vs 22.4 μmol/L; P = 0.002) and may have mild or no photosensitivity (2).

The pattern of inheritance of photosensitivity has been well documented by Went and Klasen (42) who investigated 91 families from the Netherlands, most of whom were likely to have had this type of EPP. About half the cases were sporadic. When a family contained more than one patient, affected siblings were more common than parent to child transmission. The number of families with patients in more than one generation increases with the population prevalence of the IVS3-48C allele and occurs in 15% of French and 51% of Japanese families (8). In contrast, EPP caused by FECH deficiency has not been reported in black South African families (28).

The rare patients with an IVS3-48T allele *trans* to a deleterious *FECH* mutation may also have FECH activities that are less than half-normal. This observation may be explained by the existence of rare hypomorphic alleles that remain to be identified. Such alleles may result from sequence variants in intronic and other parts

Table 1. Erythrocyte protoporphyrin concentrations and lymphocyte FECH activities in normal subjects

FECH genotype	Number of subjects	Sex	RBC protoporphyrin (nmol/L)	FECH (nmol/h/mg)
IVS3-48T/T	55	F	1484 ± 724	5.0, 4.8-5.3 (34)
	33	M	1197 ± 728	
IVS3-48C/T	53	F	1414± 763	4.2, 4.0-4.4 (32)
	33	M	1484 ± 718	
IVS3-48C/C	4	3M,1F	4610 ± 442*	2.6 (3)

FECH activities are from reference 9. Figures are means ± SD or means and 95% limits with number of subjects in parentheses. *Significantly different from IVS3-48C/T (P <0.01).

Table 2. Frequencies of FECH IVS3-48 alleles and genotypes

Country (reference)	Number of subjects	IVS3-48 *FECH* genotype (%)			Allele frequency	
		T/T	C/T	C/C	T	C
Japan (24)	104	29	52	19	55	45
Han Chinese (14)	52	39	40	21	59	41
S. E. Asia (8)	115	52	34	14	69	31
France (8)	80	77.5	22.5	0	89	11
S. Africa (European descent) (28)	93				91	9
Israel (37)	50	84	16	0	92	8
Switzerland (37)					93	7
UK (45)	100	87	13	0	93.5	6.5
Spain (10)	180				95	5
USA (31)	100	93	7	0	96.5	3.5
N. Africa (8)	75	95	5	0	97	3
Italy (1)	50				99	1
W. Africa (8)	100	99	1	0	>99	<1

of the gene that lie outside the regions normally sequenced.

FECH mutations on both alleles

FECH mutations on both alleles are an uncommon cause of EPP. To date, twenty-one symptomatic patients from 17 families have been reported (8,10,12,15,29,33,36,45) (Table 3). In the UK and France, this type of EPP accounts for about 4% of EPP families (8,45). Reported FECH activities, either measured in lymphocytes or estimated from *in vitro* expression studies, range from less than 5% to 29% of normal (median: 9%). Molecular analyses show that all patients, with the exception of 3 from consanguineous families and one other, are compound heterozygotes (Table 3). In marked contrast to the type of EPP described above, missense mutations make up 85% of the total and null mutations are unusual. Every patient carries at least one missense mutation; 4 of these (D274N, Q285R, C236Y, P168S) are present in more than one family (Table 3) but only two (P192T, D383G) are known to cause EPP when *trans* to a IVS3-48C allele alone. Most patients have the genotype IVS3-48T/T; the frequency of the IVS3-48C allele is the same as in the normal population. When the IVS3-48C allele is *cis* to a missense mutation, as in 3 patients, it will further decrease the residual activity retained by the mutation. Indeed, it is possible that some missense mutations may only become pathological through this mechanism. This distinctive pattern of mutations reflects the need to retain sufficient FECH activity for normal development, either on one allele alone or through the contribution from each allele together. Functional studies of the missense mutations identified only in this type of EPP have generally shown retention of more activity (1 – 72%, median 12% of control activity) than those identified on only one allele in EPP which, apart from P334L, have very low or undetectable FECH activities (32,35). The milder missense mutations may only cause disease in homozygotes or compound heterozygotes and thus be truly recessive; some (Y191H), like the IVS3-48C allele, may not decrease FECH activity sufficiently in homozygotes to cause photosensitivity. The C236Y mutation did not cause clinically overt EPP when *trans* to an IVS3-48C allele in one of this patient's parents indicating that this combination, and presumably similar combinations with less severe mutations, may rarely, if ever, lead to disease.

The pattern of inheritance of photosensitivity in these families is characteristic of an autosomal recessive disorder. There is an increased incidence of consanguinity, only siblings are affected and both parents are clinically normal. This last feature may in part be explained by the chance absence of a hypomorphic allele *trans* to the mutation. The mutations, P192T, D383G, IVS1+5G>A, and IVS3+2T>G, have all been reported to cause EPP when *trans* to IVS3-48C; presumably others (L72X, IVS8-2A>G, IVS10+3A>G) may also have this capacity.

Acquired somatic FECH mutations

A small number of patients have been described in whom EPP has developed after the age of 40 years in association with myelodysplasia or myeloproliferative disorder (7,34). Some of these patients have been shown to have deletions of the whole or part of chromosome 18 in haematopoietic cells, acquired as part of the myelodysplastic process and producing loss of heterozygosity for *FECH* (7,34). In only one patient has the deletion been shown to be *trans* to an IVS3-48C allele (7). This patient had particularly severe disease and acute cholestatic liver failure caused by protoporphyrin hepatotoxicity. It is unclear why loss of an allele *trans* to IVS3-48T should lead to excess formation of protoporphyrin in other patients. It may in some way be related to the abnormal erythropoiesis of myelodysplasia; deletion of an alpha-globin gene secondary to myelodysplasia also has unexpectedly severe clinical consequences (38).

ALAS2 mutations

Recently, families have been described in which EPP is inherited in an X-linked dominant pattern (43). In the UK and France, such families account for 1.6% of EPP families. Patients with this disorder have normal FECH activities but higher erythrocyte total protoporphyrin concentrations than other types of EPP of which around 40% is zinc-protoporphyrin. This high proportion of zinc-protoporphyrin suggests that protoporphyrin accumulates because supply of both its metal substrates, Fe^{2+} and Zn^{2+}, becomes rate-limiting. Additionally, the increase in erythrocyte zinc-protoporphyrin in combination with a marked increase in free protoporphyrin appears to a distinguishing feature of this form of EPP. Two frameshift mutations have been identified in 8 families that lead to predicted

Table 3. Families in whom *FECH* mutations have been identified on both alleles.

Family (reference)	FECH activity (% control)	RBC proto (µmol/L)	Allele 1	Allele 2	Phenotype*
1 (33)	19	18.6	IVS10+3A>G	G363V	LD
2 (8)	4	50	S222G, C	D274N, T	
3 (8)	10	50	P192T, T	D383G, T	
4 (15)	10	60	G55C, T	M267I, T	LD
5 (8)	6	26	D274N, T	L384S, T	
6 (8)	29	4.8	IVS8-2A>G, T	Y198H, T	
7 (45)	26**	45.7	L72X, T	F260L, T	LD
8 (29)	-	29.5	IVS1+5G>A	P62R	
9 (10)	9	32.0	A185T, T	A185T, T	LD
10 (12)	2.7**	2.3	L101P, T	IVS3+2T>G, T	KD
11 (12)	18**	2.0	Q139L, T	Q139L, T	KD
12 (12)	5.6**	16.5	P168S, T or C	Q285R, C or T	KD
13 (12)	5.9**	7.9	D274N, T	V300L, T	KD
14 (12)	6.4**	6.8	C236Y, T	D274N, T	KD
15 (12)	25**	10.5	K379N, T	C236Y, T	KD
16 (12)	-	18.5	P168S, C	P168S, C	
17 (21)	<5	4.3 – 12.0	Q285R, T	Q285R, T	KD***

*All patients were photosensitive; LD: severe protoporphyric liver disease; KD: seasonal palmar keratoderma. **Predicted from *in vitro* expression studies. ***Minor abnormalities of liver function (21). C,T: *FECH* IVS3-48C or T nucleotide *cis* to mutation. Families 1,4,11,15 contained two affected siblings; in family 1, both had severe protoporphyric liver disease.

disruption or deletion of the 19-20 C-terminal amino-acids of ALAS2. Prokaryotic expression studies show that both mutations markedly increase ALAS2 activity. Thus the C-terminal region of ALAS2 appears to inhibit enzyme activity; its removal by mutation leads to gain of function whereas all other previously described mutations in *ALAS2* decrease activity and cause hereditary sideroblastic anaemia, apart from one recently described mutation of the iron regulatory element which does not cause protoporphyria (15). Rare families with this phenotype may not have *ALAS2* mutations; the cause of their disease remains to be identified.

No mutation in FECH or ALAS2 genes

Despite advances in techniques for mutation detection (43) and the discovery of gain in function of ALAS2 as a cause of EPP (44), about 5% of EPP families are mutation-negative. Disease in these families is strongly associated with inheritance of the IVS3-48C allele and decreased FECH activity which suggests that most may have mutations in regions of the *FECH* gene that are not included in current strategies for mutation detection. At present, it is unclear whether the genotype IVS3-48C/C ever directly causes photosensitivity. Mild photosensitivity in a patient with a 4-fold increase in erythrocyte protoporphyrin concentration, normal plasma porphyrin concentration and no *FECH* mutation, has recently been ascribed, at least partly, to this genotype (37). Five of 191 unrelated EPP patients in the UK had the genotype IVS3-48C/C; in only one of these was no mutation identified. Because FECH is a homodimer, it is possible that a mutation on one allele may exert a dominant-negative effect and cause protoporphyria when *trans* to a normal allele. In mice, deletion of intron 10 acts in this way and *in vitro* some wild-type/EPP mutant heterodimers have lower than predicted FECH activities (19,23). However, no family with the expected autosomal dominant EPP that would be produced by this mechanism has yet been described, though the possibility that it might it might act with the IVS3-48C allele to decrease activity in some patients cannot be excluded.

GENOTYPE: PHENOTYPE CORRELATIONS

The cutaneous features of EPP, though varying in severity are remarkably uniform (11). No correlation between indices of severity of photosensitivity, such as age of onset or duration of symptoms, and genotype have yet been reported apart from the suggestion that the relatively common missense mutation , P334L, may cause only mild disease (36). Apart from this instance, no correlation between erythrocyte protoporphyrin concentration and type of *FECH* mutation has been reported (2). In general, establishing correlation between individual mutations and clinical features is complicated by the difficulty of collecting sufficient unrelated patients who share the same mutation. On the other hand, a correlation has been established between one molecular form of EPP and a cutaneous feature. Thus, seasonal palmar keratoderma has to date been reported only in patients who are compound heterozygotes or homozygotes for *FECH* mutations (13,21) (Table 3).

Severe liver disease, often requiring treatment by transplantation, occurs in about 2% of patients with EPP. Patients who have *FECH* mutations on both alleles (Table 3) or a gain of function mutation of *ALAS2* (43) have an increased risk of liver disease though together they account for only a small proportion of those with liver disease. The risk is high in patients with *ALAS2* mutations, as are erythrocyte protoporphyrin concentrations, whereas protoporphyric liver disease has not yet been reported in patients with palmar keratoderma; a group of patients in whom erythrocyte protoporphyrin concentrations are strikingly lower than in other types of EPP. Among patients with *FECH* mutations on both alleles, 5 (42%) of 12 reported patients without keratoderma have had liver disease. However, this high prevalence may in part be due to reporting bias and data on larger numbers of these very rare patients is required.

In patients with liver disease and a *FECH* mutation on one allele, FECH activity is particularly low in liver and lymphoblasts compared to patients without liver disease (3,5) decreasing to levels comparable to those reported for patients with *FECH* mutations on both alleles (8). Investigation of the *FECH* locus has not explained this observation. In one family, hypermethylation of *FECH* was proposed as a mechanism for an additional decrease in *FECH* expression (27) but this has not yet been confirmed in other families. A mainly literature-based study of 112 patients with a single *FECH* mutation, of whom 18 had severe liver disease, found that all patients with liver disease had a ‘null’ mutation (splicing defect, nonsense or

frameshift) while none of the 20 patients with a missense mutation had liver disease; this difference was statistically significant (22). Under-representation of missense mutations was also noted in a group of 15 patients with liver disease, all studied in the same laboratory, but comparison with a group without liver disease did not reach statistical significance (5,3). Together these studies suggest that missense mutations that preserve significant amounts of residual activity may carry a lower risk of liver disease than 'null' mutations, a group that includes those misssense mutations that abolish FECH activity. However, 'null' mutations associated with liver disease are found more frequently in patients without liver disease; an observation that indicates the probable importance of genetic factors outside the *FECH* locus and acquired factors in the pathogenesis of protoporphyric liver disease. Mutational analysis currently has no role in predicting the risk of liver disease in an individual patient except when it leads to identification of mutations on both *FECH* alleles or an *ALAS2* mutation.

Inheritance and nomenclature

The investigations described above identify two different forms of EPP: one caused by FECH deficiency and the other by gain of function mutations in *ALAS2*. The latter has distinctive biochemical and genetic characteristics and has been named X-linked dominant protoporphyria (MIM 300752). Nomenclature of the more common forms of EPP caused by *FECH* mutations leading to FECH deficiency (MIM 177000) is less straightforward. This type of EPP was long regarded as an autosomal dominant disorder with incomplete penetrance and the term 'dominant EPP' has been widely used to distinguish patients with a *FECH* mutation trans to a hypomorphic IVS3-48C allele from those with 'autosomal recessive' EPP who are hetero- or homoallelic for *FECH* mutations. This distinction and use of the term 'dominant' has recently been criticized because the molecular data shows that FECH-deficient EPP is always inherited as an autosomal recessive trait (49). All patients are compound heterozygotes or, rarely, homozygotes for loss of function *FECH* variants, at least one of which must have sufficient residual activity to sustain normal development. Because one of these alleles (IVS3-48C) is common, families may show pseudodominant inheritance as in other autosomal recessive diseases in which a hypomorphic allele is common. In EPP, the proportion of families showing pseudodominant inheritance increases with the population frequency of the hypomorphic allele. In principle, families with patients who have loss of function *FECH* variants, other than IVS3-48C, on both alleles may also show pseudodominant inheritance although this is rare because it requires co-inheritance of a IVS3-48C allele with a variant that abolishes or markedly decreases FECH activity; photosensitivity in more than generation has been reported in only one family (12). In spite of this uniformity at the molecular level, in practice phenotypic differences, particularly in relation to the risk of liver disease, make it useful to distinguish between compound heterozygotes in whom one allele is IVS3-48C and compound heterozygotes or homozygotes for other *FECH* variants. It therefore seems reasonable to use the term 'pseudominant EPP' for the former and retain the term 'autosomal recessive EPP' for the latter while bearing in mind the compromise that this entails.

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