Gene Dosage Analysis Identifies Large Deletions of the *FECH* Gene in 10% of Families with Erythropoietic Protoporphyria

Sharon D. Whatley[1], Nicola G. Mason[1], S. Alexander Holme[2], Alex V. Anstey[3], George H. Elder[1] and Michael N. Badminton[1]

Erythropoietic protoporphyria (EPP) is an inherited cutaneous porphyria characterized by partial deficiency of ferrochelatase (FECH), accumulation of protoporphyrin IX in erythrocytes, skin, and liver, and acute photosensitivity. Genetic counseling in EPP requires identification of *FECH* mutations, but current sequencing-based procedures fail to detect mutations in about one in six families. We have used gene dosage analysis by quantitative PCR to identify large deletions of the *FECH* gene in 19 (58%) of 33 unrelated UK patients with EPP in whom mutations could not be detected by sequencing. Seven deletions were identified, six of which were previously unreported. Breakpoints were identified for six deletions (c.1–7887–IVS1 + 2425insTTCA; c.1–9629–IVS1 + 2437; IVS2−1987–IVS4 + 352del; c.768–IVS7 + 244del; IVS7 + 2784–IVS9 + 108del; IVS6 + 2350–TGA + 95del). Five breakpoints were in intronic repeat sequences (AluSc, AluSq, AluSx, L1MC4). The remaining deletion (Del Ex3–4) is likely to be a large insertion–deletion. Combining quantitative PCR with routine sequencing increased the sensitivity of mutation detection in 189 unrelated UK patients with EPP from 83% (95% CI: 76–87%) to 93% (CI: 88–96%) ($P = 0.003$). Our findings show that large deletions of the *FECH* gene are an important cause of EPP. Gene dosage analysis should be incorporated into routine procedures for mutation detection in EPP.

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INTRODUCTION

Erythropoietic protoporphyria (EPP) (OMIM 177,000) is an inherited cutaneous porphyria caused by partial deficiency of ferrochelatase (FECH) (EC 4.99.1.1), the final enzyme in the heme biosynthetic pathway, which catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. Deficiency of FECH leads to accumulation of protoporphyrin IX in erythrocytes, plasma, skin, and liver. Accumulation of protoporphyrin in the skin produces lifelong acute photosensitivity while its deposition in the liver leads to hepatobiliary disease in some patients (Todd, 1994; Meerman, 2000).

The inheritance of EPP is complex. In most families, clinical expression requires inheritance of a hypomorphic *FECH* IVS3-48C *trans* to a disabling *FECH* mutation that is inherited in an autosomal dominant fashion (Gouya *et al.*, 2006) but rare families with autosomal recessive EPP have been reported (Lamoril *et al.*, 1991; Sarkany *et al.*, 1994; Poh-Fitzpatrick *et al.*, 2002; Whatley *et al.*, 2004; Gouya *et al.*, 2006). The frequency of the hypomorphic *FECH* allele varies between populations (Gouya *et al.*, 2006), being present in about 12% of white Europeans (Gouya *et al.*, 2002, 2006; Whatley *et al.*, 2004) but in 68% of Japanese (Saruwatari *et al.*, 2006). Molecular analysis to identify the IVS3-48C allele and disease-specific mutations in the *FECH* gene is important for accurate genetic counseling of families with EPP (Gouya *et al.*, 2002).

Over 120 mutations in the *FECH* gene, which contains 11 exons spread over 45 kb, have been reported in EPP, most of which are restricted to one or a few families (Gouya *et al.*, 2006; Saruwatari *et al.*, 2006; Aurizi *et al.*, 2007). Apart from one complete gene deletion (Magness *et al.*, 1994) and a partial deletion (Wood *et al.*, 2006), all have been detected using methods that depend only on sequencing of exons and their flanking regions, including up to 1.3 kb of 5′ non-coding sequence, to identify mutations. This approach fails to identify an *FECH* mutation in about one in six families (Rüfenacht *et al.*, 1998; Whatley *et al.*, 2004; Gouya *et al.*, 2006). Here we show that gene dosage analysis by quantitative PCR detects gross deletions of one or more *FECH* exons in 19 of 33 (58%) of such ''mutation-negative'' families and that deletions of this type are present in 10% of UK families with EPP.

[1]*Department of Medical Biochemistry and Immunology, University Hospital of Wales and School of Medicine, Cardiff University, Cardiff, UK;* [2]*Department of Dermatology, Queen Margaret Hospital, Dumfermline, UK and* [3]*Department of Dermatology, Cardiff University, Cardiff, UK*

Correspondence: Dr Sharon D. Whatley, Department of Medical Biochemistry and Immunology, University Hospital of Wales, Heath Park, Cardiff CF14 4XW, UK. Email: whatley@cardiff.ac.uk

Abbreviations: EPP, erythropoietic protoporphyria; FECH, ferrochelatase; WT, wild type

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RESULTS

Direct sequencing of all exons, 30–250 bp of intronic flanking regions, and 1,000 kb of 5′ untranslated region (UTR) identified an *FECH* mutation on one or both alleles in 156 (83%) of our 189 unrelated patients, 149 of whom had a mutation on only one allele. Genotyping of the 33 mutation-negative patients showed that 28 (85%) patients carried an *FECH* IVS3-48C allele, a significantly lower frequency than in the 149 patients who were heterozygous for an *FECH* mutation, all but one of whom had at least one IVS3-48C allele (χ^2 13.3; $P<0.005$).

Gene dosage analysis by quantitative PCR of genomic DNA from the 33 mutation-negative patients identified deletions encompassing one or more exons in 19 (58%) patients, 18 of whom were either *FECH* IVS3-48C/T heterozygotes or had the IVS3-48C allele *trans* to a deletion (Table 1). Seven different deletions were detected, one of which has been described previously (Wood *et al.*, 2006) (Table 1). Exon 1 was deleted in five patients; genotyping with three highly informative microsatellite markers, two flanking the *FECH* gene and one in intron 4, identified two haplotypes that differed only at the 5′ flanking site. Two different deletions were identified: three patients had a 10,379 bp deletion with a small 4 bp insertion and two patients had a 12,133 bp deletion (Table 1). Two patients had lost exons 3 and 4; one has previously been described (Wood *et al.*, 2006), the other had a deletion for which breakpoints were not identified (Table 1). In two patients, a small deletion involving part of exon 7 and 244 bp of 3′ flanking sequence removed the region in which one of the primers for amplification of this exon is usually based. The presence of a stop codon 68 codons 3′ to this deletion suggests that any mRNA produced from this allele will be truncated and unstable. Exons 8 and 9 were deleted in eight patients; genotyping with three microsatellite markers showed that this mutation was present in at least two different *FECH* haplotypes. The largest deletion removed exons 7, 8, 9, 10, and 11 and was present in two patients.

Breakpoints for six deletions were defined by sequencing (Table 2). The 10.4 kb exon 1 deletion contained a four-base insert with a sequence that was repeated in the 5′ flanking region (Table 2) and 30 bp 3′ to the insertion. For four of the five other deletions, the breakpoints shared the same 3, 4, or 5 bp sequences at the deletion junctions (Table 2). The 3′ breakpoints of both exon 1 deletion and the exon 7 deletion and the 5′ breakpoint of the exon 8–9 deletion were all sited in Alu repeat regions while the 5′ breakpoint of the exon 7–11 deletion was in an L1MC4 repeat sequence (Table 2). These features have been reported for large deletions in other genes and are believed to facilitate homologous recombination (Woods-Samuels *et al.*, 1991; Laccone *et al.*, 2004).

We were unable to identify the breakpoint for one deletion. This patient was heterozygous for a deletion that included exons 3 and 4 and surrounding sequence from IVS2-88 to IVS4 + 2046 and thus differed from the exon 3–4 deletion with defined breakpoints recently described in two Italian families (Di Pierro *et al.*, 2007). PCR amplification using primers flanking the deleted region gave a 7 kb band consistent with the size of the deletion as identified by gene dosage analysis. No smaller product was identified using amplification conditions that are capable of detecting fragments as small as 100 bp. Restriction enzyme (*Nde*I) digestion of the 7 kb amplicon gave a normal pattern consistent with the amplicon coming from the normal allele alone. These findings exclude the presence of a 7 kb insertion–deletion but not of an insertion–deletion too large to amplify.

DISCUSSION

Most patients with EPP are heterozygous for disabling mutations in the *FECH* gene (Gouya *et al.*, 2006). Intragenic deletions involving entire exons and their flanking regions, including primer binding sites, are not identified in heterozygotes by methods for mutation detection that rely on direct sequencing of amplified genomic DNA because sequencing does not distinguish between amplification of one or both alleles. Quantitative analysis of amplified genomic DNA is required for this purpose. Here, we have used gene dosage analysis to show that large deletions of the *FECH* gene, likely to abolish all FECH activity, were present in 19 (58%) of 33 patients with EPP in whom *FECH* mutations were not identified by sequencing.

Such deletions are an important cause of human disease and represent 5.6% of all mutations in the human gene mutation database (www.hgmd.cf.ac.uk). There is evidence that they result from various molecular interactions that are

Table 1. Deletions in the *FECH* gene in patients with EPP

Number of patients	Deleted exons	Deletion	Size of deletion (bp)	*FECH* IVS3-48 genotypes
3	Exon 1	c.1–7887 to IVS1+2425insTTCA	10,379	C/T
2	Exon 1	c.1–9629 to IVS1+2437	12,133	C/T
1[1]	Exons 3, 4	IVS2-1987 to IVS4+352	4,425	C/–
1	Exons 3, 4	See text	>7,000	C/–
2	Exon 7 (partial)	c.768 to IVS7+244	280	C/T, T/T
8	Exons 8, 9	IVS7+2784 to IVS9+108	2,196	C/T
2	Exons 7–11	IVS6+2350 to TGA+95	9,889	C/T

[1]Wood *et al.* (2006).

Table 2. Deletion breakpoints in the *FECH* gene

Deleted exon(s)	Sequence	Breakpoints	Repeat sequence
1 (10.4 kb)	Junctional	gttcacgcaca*TTCA*ggctcactgca	
	5′WT	gtgcattcaggttcacgcaca	—
	3′WT	ggctcactgcaacctccgcct	AluSc
1 (12.1 kb)	Junctional	tttgctgttgtTGCCCggcctgtttgt	
	5′WT	tacacagagttttgctgttgtTGCCC	—
	3′WT	TGCCCggcctgtttgtaggccttcta	AluSq
3, 4[1]	Junctional	5′ ctttagttttcGAGgctgctgctat 3′	
	5′WT	atcatagtatctttagttttcGAG	—
	3′WT	GAGgctgctgctattattgccttt	—
7	Junctional	5′agagaagcgagGTGtcacccaggct 3′	
	5′WT	ccacttgagaagagaagcgagGTG	—
	3′ WT	GTGtcacccaggctggagtgcaat	AluSx
8, 9	Junctional	5′ ctatttttctcTTTGcatgtacggt 3′	
	5′WT	ctaaaattactatttttctcTTTG	AluSx
	3′WT	TTTGcatgtacggtgtcctcattc	—
7, 8, 9, 10, 11	Junctional	5′ tgggaccacag||catccttcctt 3′	
	5′WT	tgctgagtagctgggaccaca	L1MC4
	3′ WT	catccttccttgatatatata	—

Wild-type (WT) sequences 5′ and 3′ to the breakpoints are shown. Junction points, except for the flush junction indicated by ||, and homologous sequences present at both the 5′ and 3′ breakpoints are in capitals. The inserted sequence is indicated by italic capitals.
[1]Previously reported (Wood *et al.*, 2006).

dependent on the features of the surrounding DNA sequence (Abeysinghe *et al.*, 2006). Repetitive elements, which were present in all but one of the deletions for which we defined breakpoints, are known to facilitate the formation of secondary structures such as hairpin loops and cruciforms (non-B DNA conformations) that have been shown to coincide with the breakpoints of gross deletions (Chuzhanova *et al.*, 2003; Bacolla *et al.*, 2004).

The 33 patients came from a cohort of 189 apparently unrelated patients identified by a cross-sectional study of EPP in the UK (Holme *et al.*, 2006). *FECH* mutations were identified by sequencing in 156 (83%) of these 189 patients. Here we show that a further 19 (10%) patients had large intragenic deletions. Thus sequencing combined with gene dosage analysis identifies mutations in 175 of UK patients with EPP, increasing the sensitivity of mutation detection to 93% (95% confidence interval: 88–96%) (χ^2 8.7; $P=0.003$), which is higher than those reported previously (Rüfenacht *et al.*, 1998; Whatley *et al.*, 2004; Gouya *et al.*, 2006).

Only one of the 19 patients with deletions was not heterozygous for the hypomorphic *FECH* IVS3-48C allele (Table 1). This patient had the smallest deletion that we identified as involving exon 7 and its 3′ flanking region (Tables 1 and 2). However, it is unlikely that the mutant allele gives rise to a stable truncated protein (see Results) capable of exerting a dominant-negative effect on FECH activity, as reported for an exon 10 deletion in the mouse (Magness *et al.*, 2002). At present, it is not clear why rare patients with the dominant form of EPP who have a mutation *trans* to a normal *FECH* IVS3-48T allele develop photosensitivity (Wiman *et al.*, 2003).

Sequencing combined with gene dosage analysis failed to identify an *FECH* mutation in 14 (7%) of UK patients with EPP. Ten of these patients were heterozygous for the hypomorphic *FECH* IVS3-48C allele, a proportion that is significantly higher than in the UK population (χ^2 26.0, $P<0.001$). This suggests that most, if not all, of these 10 patients may be heterozygous for deleterious *FECH* mutations that lie in regions of the gene that determine expression but are outside the regions that we sequenced. The other four patients all had the genotype *FECH* IVS3-48T/T, a proportion that is much higher than that in other groups of EPP patients (Risheq *et al.*, 2003; Gouya *et al.*, 2006) except for those with mutations on both *FECH* alleles (Whatley *et al.*, 2004; Gouya *et al.*, 2006). It seems unlikely that these patients are homozygous or compound heterozygotes for *FECH* mutations that were not detected by our methods since all patients with recessive EPP yet reported have had at least one intra-exonic mutation (Whatley *et al.*, 2004; Gouya *et al.*, 2006). Alternatively, at least some of these patients may have mutations in genes other than *FECH*. Deletion of the Irp2 gene has recently been shown to produce protoporphyria in mice (Cooperman *et al.*, 2006), and the possibility that EPP may also be caused by mutation of genes that regulate the supply of substrates to FECH remains to be excluded.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 189 apparently unrelated patients with EPP as part of a cross-sectional study of EPP in the

UK (Holme *et al.*, 2006). We investigated 33 of these patients (32 white British, one of Iraqi descent) in whom an *FECH* mutation could not be identified by direct sequencing of all exons, 30–250 bp of intronic flanking regions, and 1,000 kb of 5′ UTR. Total erythrocyte porphyrin was increased in all 33 patients (mean 25.6 μmol/l, range 6.2–65.7 μmol/l; reference range 0.4–1.7 μmol/l) and was mainly free protoporphyrin.

The study was conducted in accordance with the Declaration of Helsinki Principles for medical research involving human subjects and its subsequent amendments. Prior approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics committees. All patients or their parents gave informed consent.

Preparation of DNA

Genomic DNA was extracted from whole blood using the QIAamp DNA purification kit (Qiagen, Crawley, UK) for sequencing and the Flexigene kit (Qiagen) for dosage studies.

Gene dosage analysis

A multiplex PCR was designed to simultaneously amplify exons 2–9 and 11 of the *FECH* gene, along with exon 9 of the *HMBS* gene and exon 5 of the *PPOX* gene as internal controls. Exons 1 and 10 did not amplify consistently within the multiplex, so they were analyzed separately with exon 1 of the *UROS* gene and exon 4 of the *HMBS* gene as internal controls. A fluorescent (5′ FAM) label was incorporated into one of each primer pair. Details of the primers used for gene dosage analysis are available from the authors. Amplification was carried out in 20 μl volumes containing 5.39 μl DNA, 3.45 μl primer mix (0.5–1.5 pmol (final concentration) of each primer), and 10 μl 2 × Qiagen multiplex PCR master mix (containing HotStarTaq DNA polymerase, Qiagen multiplex PCR buffer (6 mM $MgCl_2$, pH 8.7), dNTP mix). An initial denaturation of 15 minutes at 95°C was followed by 20 cycles of denaturation for 30 seconds at 94°C, annealing for 1 minute 30 seconds at 64°C, and extension for 1 minute 30 seconds at 72°C, with a final extension for 10 minutes at 72°C. The PCR products (2 μl) were added to a mixture containing formamide (10 μl) and GS500 standard (0.3 μl) (PE Applied Biosystems, Cheshire, UK) and denatured at 95°C for 3 minutes. The samples were run on an ABI Prism 310 analyser at 60°C using POP4 polymer (PE Applied Biosystems).

To determine the gene dosage for each exon, the peak areas were compared with each other and against controls (Yau *et al.*, 1996). A locus with a double copy will give a theoretical value of 1.0 whereas a locus with a deletion will give a value of 0.5 and 2.0 depending on whether the deleted locus is the numerator or the denominator. To exclude the presence of polymorphisms at the primer sites, all deletions were confirmed using at least one other set of primers to eliminate false positive results due to mis-priming. Primer sequences are available from the authors.

Identification of breakpoints

To identify deletion breakpoints, pairs of primers were designed for quantitative PCR-amplification of intronic regions around the deleted exon; these primer sequences are available from the authors. These amplicons were multiplexed with internal controls and analyzed for dosage. The results indicated whether each amplicon was within the deleted region or not. Pairs of primers were redesigned at increasing or decreasing distance from the deletion, depending on these results, partially localizing the deletion. Where possible, the forward primer from a diallelic region 5′ of the deletion was used with the antisense primer from a diallelic region 3′ of the deletion to amplify a PCR product straddling the deletion. This amplicon was sequenced and the breakpoints were identified. Amplification across breakpoints was carried out using Elongase enzyme mix (Invitrogen, Paisley, UK) according to the manufacturer's instructions, using a final concentration of 1.8 mM magnesium with an elongation time between 5 and 20 minutes.

Haplotype analysis

Genotyping with two microsatellite markers flanking the *FECH* gene (315.0 kb 5′ to the start codon, 267.6 kb 3′ to the stop codon) and with one microsatellite marker in intron 4 was carried out as described by Parker (2006).

Sequencing of genomic DNA

PCR-amplified double-stranded DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen) before being cycle sequenced using fluorescent ddNTPs (BigDye) and an ABI Prism 3100 Genetic Analyzer (PE Biosystems, Warrington, UK). Nucleotides are numbered from the cDNA sequence of human *FECH* (GenBank accession number D00726) with the A of the ATG initiation codon as "+1".

Other methods

Erythrocyte porphyrins were analyzed as described (Deacon and Elder, 2001).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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