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SHORT REPORT

Biochemical and molecular diagnosis of erythropoietic protoporphyria in an Ashkenazi Jewish family

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Summary Erythropoietic protoporphyria (EPP) is a rare hereditary disorder due to a partial deficiency of ferrochelatase (FECH). The genotype of EPP patients features a mutation on one allele of the *FECH* gene and a common hypomorphic *FECH* IVS3–48c on the other allele (M/c). The resulting enzyme activity in patients is ~35% of that in normal individuals. Ferrochelatase deficiency results in the accumulation of protoporphyrin in the skin, which is responsible for the clinical symptom of cutaneous photosensitivity in patients. In this study, we report the identification of a novel *FECH* mutation *del*T23 in an 11-member EPP family of Jewish origin. Two EPP siblings shared an identical genotype of *del*T23/IVS3–48c (M/c). They were both photosensitive and showed highly increased erythrocyte protoporphyrin. The genotype of the patients' mother, who did not present with any EPP clinical symptoms, was *del*T23/IVS3–48t (M/t). The patients' father, an offspring of consanguineous parents, was homozygous IVS3–48 c/c. He exhibited a mild photosensitivity, and an increase of 4-fold in erythrocyte protoporphyrin. His FECH mRNA amount was 71% of that of genotype t/t. It is the first reported case of an individual with c/c genotype who exhibits both biochemical and clinical indications of EPP. These results suggest that IVS3–48c is a functional variant of ferrochelatase. The clinical symptoms and biochemical abnormalities in the patients' father could be the result of an interaction between genetic and environmental factors. In addition, the frequency of IVS3–48c in the Ashkenazi Jewish population was estimated at 8%, which is similar to that in the European populations.

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References to electronic databases: OMIM: http://www.ncbi.nlm.nih.gov/Omim/. Gen Bank: http://www.ncbi.nlm.nih.gov/GenBank/ (for human FECH cDNA [NM000140], human FECH gene [NT025028], human FECH protein [NP000131]). Human Gene Mutation Database (HGMD): http://www.archive.uwcm.ac.uk/uwcm/mg/hgmd0.html/. Erythropoietic protoporphyria: OMIM #177000). Ferrochelatase: EC 4.99.1.1.

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Abbreviations

EPP	erythropoietic protoporphyria
FECH	ferrochelatase
PCR	polymerase chain reaction
RBC	red blood cells
SNP	single nucleotide polymorphism

Introduction

Erythropoietic protoporphyria (EPP, OMIM #177000) is a hereditary disorder of the haem biosynthetic pathway. The affected enzyme ferrochelatase (FECH,

EC 4.99.1.1) catalyses the insertion of iron into protoporphyrin IX to form haem. As the result of ferrochelatase deficiency, a large amount of protoporphyrin accumulates in the skin, which is responsible for the clinical symptoms of photosensitivity in EPP patients (Anderson et al 2001). In addition to the cutaneous symptoms, EPP-related liver failure occurs in about 1% of the patients (Holme et al 2006). EPP is transmitted as an autosomal dominant trait in >97% of the cases, or as an autosomal recessive trait. In the dominant inheritance, which occurs in the majority of the families, the clinical expression of EPP requires a deleterious mutation on one allele of the *FECH* gene and a common SNP IVS3–48c on the other allele (M/c). The frequencies of IVS3–48c vary from population to population. The highest frequency found so far, 45%, was in the Japanese population and the lowest, less than 1%, among black Africans (Gouya et al 2006; Nakano et al 2006).

To date, a total of 109 mutations have been described in the *FECH* gene of EPP families (HGMD, accessed in March 2008). In this study, we report biochemical and genetic characterization of EPP in an Ashkenazi Jewish family in which the father was an offspring of consanguineous parents.

Material and methods

Subjects and biochemical analyses

An 11-member Ashkenazi family of three generations was studied (Fig. 1). The index patient, a 36-year-old woman and her 26-year-old brother, have suffered from skin photosensitivity since early childhood. They were diagnosed as EPP patients at the ages of 24 and 14 years, respectively. The diagnosis was established in both on the basis of highly increased erythrocyte protoporphyrin and a distinct peak in the plasma emission scan at 635 nm (Table 1). As shown in the table, both exhibited increased faecal protoporphyrin. Normal faecal coproporphyrin (15 and 25 μg/g dry weight, respectively) and a ratio of 0.3 of coproporphyrin III/I (normal <1) were measured in both of them (not shown). So far, the patients have exhibited normal liver function. The father of the index patient, who was an offspring of consanguineous parents, suffered from mild symptoms of photosensitivity. He experienced a feeling of itching and burning in the skin shortly after sun exposure. He has tried to avoid the sun throughout his life. Repeated measurements showed 3- to 4-fold increases in erythrocyte protoporphyrin concentration. A slight increase in erythrocyte protoporphyrin concentration was observed also in the mother as well as in the eldest son of the index patient (Table 1). The rest of the family members were not tested biochemically. None of them showed clinical signs of EPP.

Fifty non-porphyric individuals of Jewish origin (Ashkenazi), three non-porphyric caucasians with genotypes t/t, c/t and c/c at IVS3–48 in the *FECH* gene, respectively, as well as an EPP patient from an unrelated family (genotype M/c), were recruited into the study. Informed consent was obtained from all subjects. This study was approved by the Helsinki committee of the Israeli Ministry of Health.

Plasma fluorescence emission scanning was carried out according to Long et al (1993). Erythrocyte free

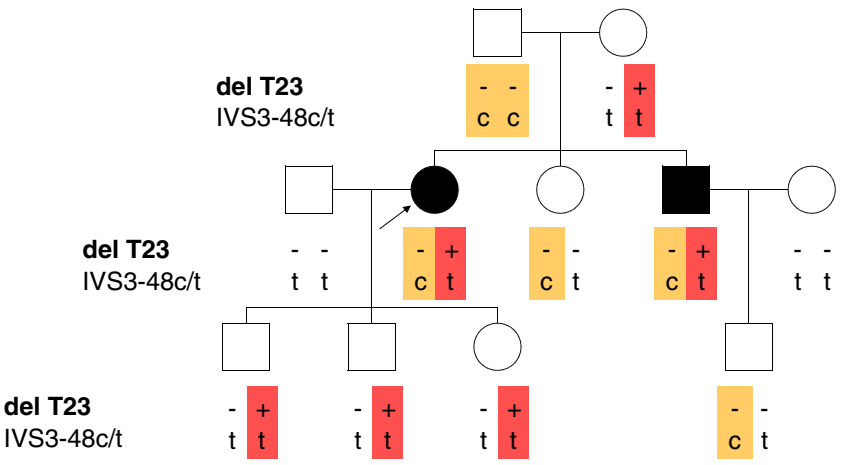

Fig. 1 Pedigree of an Ashkenazi EPP family. Solid symbols represent EPP patients with the genotype M/c and the open symbols represent individuals with genotype M/t. The index patient is indicated by an arrow. The *FECH* alleles are illustrated below the symbol of each individual. The mutated allele is highlighted in *red* and the low-expressed allele (IVS3–48c) in *orange*. wt, wild type; M, mutation *del*T23. The presence/absence of mutation *del*T23 is indicated by '+/−'

Table 1 Clinical and biochemical data from the Israeli EPP family

Subjects	Age (years)	Photosensitivity	Erythrocyte protoporphyrin (<80 μg/dl RBC)	Plasma fluorescence peak (mm)[a]	Faecal protoporphyrin (<80 μg/g dry weight)	Mutation *del*T23	SNP IVS3-48c/t
Index[b]	36	+++	4917	188	680	Present	c/t
Brother[b]	26	+++	6684	128	1072	Present	c/t
Father	58	+	338	2	<80	Absent	c/c
Mother	58	–	133	4	<80	Present	t/t
Sister	33	–	70	None	<80	Absent	c/t
Eldest son	9	–	160	3	n.d.	Present	t/t

n.d.: not determined.
[a] Height of the peak in the emission scan, observed at wavelengths 404 nm (excitation) and 635 nm (emission).
[b] Biochemical analyses were carried out at the time of diagnosis.

protoporphyrin was measured according to the method of Piomelli (1973), modified as previously described (Schoenfeld et al 2003). Faecal porphyrins were extracted according to With (1978) and separated by HPLC according to Lim and Peters (1984).

Mutational analysis

Peripheral blood samples were collected from all subjects. Genomic DNA was isolated from peripheral blood using the QIAamp Blood Kit (Qiagen, Hilden, Germany). The 11 coding exons of the *FECH* gene, along with 52–191 bp of their flanking regions, were amplified by PCR using specific primers. Sequence analysis was performed on the ABI Prism 310 genetic analyser (Applied Biosystems, Foster City, CA, USA). Sequence analysis of genomic DNA also revealed the status of SNP IVS3–48c/t in the subjects.

Preparation of cDNA

For preparation of cDNA, a separate blood sample was collected from the subjects concerned in a PAXgene Blood RNA tube (Qiagen). Total RNA was isolated by using the PAXgene Blood RNA Validation Kit (Qiagen). An aliquot of total RNA was analysed on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) using the Eukaryote Total RNA Nano Kit. The quality of total RNA was evaluated by both the 28S/18S ratio and RIN (RNA integrity number). mRNA (1 μg of total RNA) was reverse-transcribed into cDNA by using the First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland).

Real-time PCR quantification of FECH cDNA

Real-time PCR quantification of ferrochelatase cDNA was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). β-Actin was used as an endogenous control. Reagents used for real-time PCR were all purchased from Applied Biosystems. PCR conditions, i.e. reaction mixture and temperature profile, were according to the manufacturer's recommendations. The relative amount of FECH mRNA was calculated as previously described (Aurizi et al 2007).

Genotyping using microsatellite markers

Genotype analysis was conducted in the father of the index patient using 10 microsatellite markers. These markers are located in the genomic region of the *FECH* gene in the order of cen-D18S69-D18S1152-D18S41-D18S858-**FECH**-D18S381-D18S977-D18S1144-D18S1129-D18S1155-D18S64-qter in the LDB map (Collins et al 1996). Fluorescence-labelled PCR primers were synthesized with the available sequence information. PCR was carried out using standard conditions. Fragment length analysis was performed on the ABI Prism 310 genetic analyser (Applied Biosystems).

Results

Mutational analysis was initiated in the index patient. Direct sequencing of PCR-amplified *FECH* gene fragments identified a single nucleotide T deletion at cDNA position 23 in the first exon (*del*T23). Frameshift, as the result of the deletion, generates a premature stop codon at amino acid position 72. The rest of the family members were screened specifically for the *del*T23 mutation. The result showed that besides the index patient, five other individuals including her brother, her mother and all three of her children also carried the mutation. No mutation was

Table 2 Levels of FECH mRNA in various individuals

Subjects	SNP IVS3-48c/t	Amount of normal *FECH* transcript in relation to that in control 1 (defined as 100%)
Control 1	t/t	100
Control 2	c/t	93
Control 3	c/c	64
Father of the index patient	c/c	71
An unrelated EPP patient	M/c	32

found in the entire *FECH* gene of the patients' father by sequencing. Mutation *del*T23 was also absent in the *FECH* gene of 50 non-porphyric Ashkenazi subjects.

SNP IVS3–48c/t was analysed among all the members of the family. The segregation pattern between mutation delT23 and SNP IVS3–48c/t within the family is depicted in Fig. 1. Genotyping of SNP IVS3–48 among 50 non-porphyric Ashkenazi control subjects identified 42 homozygotes IVS3–48t/t and 8 heterozygotes IVS3–48c/t. The frequency of IVS3–48c is therefore estimated as 8% in this population.

The father of the index patient was homozygous IVS3–48c/c, a rare genotype in the general population. The homozygosity was mostly likely due to the parental consanguinity. Besides IVS3–48c/c, he was homozygous for other intragenic SNPs including IVS1–23t/t, 287G/G, 798C/C, 921G/G, IVS10–61g/g. The results of microsatellite analysis extended the homozygosity to a distance of at least 7.4 cM flanking the *FECH* gene.

To test the presence of diploid *FECH* alleles in the patients' father, FECH mRNA was quantified by real-time PCR and compared with that of control subjects (genotypes t/t, c/t and c/c), as well as that of an EPP patient from an unrelated family (genotype M/c). As shown in Table 2, the relative amount of FECH mRNA decreases in the order of t/t, c/t, c/c and M/c. The value obtained from the father of the index patient was 71% that of control 1 (genotype t/t), which is comparable with 64% measured in control 3 (genotype c/c). However, control 3 was non-porphyric.

Discussion

EPP cases have been described in various populations. The family of this study however, is the second genetically tested EPP family ever reported in Israel (Schoenfeld et al 2003). Extensive biochemical analyses were conducted among members of this family prior to genetic testing. The clinical and the biochemical evidences, which included cutaneous photosensitivity in combination with immense increases of protoporphyrin concentration in erythrocytes as well as presence of fluorescence peaks at 635 nm, was sufficient to ascertain an EPP diagnosis in the index patient and her brother. Interestingly, both of their parents also presented with abnormal erythrocyte protoporphyrin concentrations, i.e. a 4-fold increase in the father and a 1.7-fold increase in the mother, and measurable peaks in the plasma scan (Table 1). In addition, the father was slightly photosensitive. These findings raised the question whether EPP is recessive in this family.

At the genetic level, the identification of a single mutation *del*T23 and the characterization of SNP IVS3–48c/t in the *FECH* gene proved the dominant form of EPP in the family. As shown in Fig. 1, both patients have an identical genotype of a mutated *FECH* allele *in trans* to a low-expressed *FECH* allele featuring IVS3–48c (M/c). This genotype, according to Gouya et al, is required for the clinically *overt* EPP which is the result of a reduction of the overall enzyme activity below a critical threshold of ~35%, assuming that the allele in which the frameshift mutation *del*T23 is resided will not produce any active enzyme (Gouya et al 2006). The *del*T23 mutation apparently originated from the patients' mother. She, as well as three of her grandchildren, had the genotype of a mutated allele *in trans* to a normal *FECH* allele (M/t). According to the current knowledge, these four individuals will remain asymptomatic throughout their lives because of the 50% of normal amount of ferrochelatase enzyme they possess.

A more complex situation was found in the patients' father. Clinically, he presented with only a mild

Table 3 Frequency of low-expressed *FECH* allele (IVS3–48c) among different populations

Population	Frequency	Source
Japanese	45%	Nakano et al (2006)
Chinese (Han)	41%	Kong et al (2008)
Southeast Asian	31%	Gouya et al (2006)
British	13%	Berroeta et al (2007)
French	11%	Gouya et al (2006)
Jewish (Ashkenazi)	**8%**	**This study**
Swiss	7%	Schneider-Yin and Minder, unpublished data
Spanish	5%	Herrero et al (2007)
North African	3%	Gouya et al (2006)
Italian	1%	Aurizi et al (2007)
Black West Africa	<1%	Gouya et al (2006)

photosensitivity. Biochemically, a substantial increase in protoporphyrin concentration was repeatedly measured in erythrocytes. At the genetic level, no mutations were identified in his *FECH* gene apart from the homozygosity of IVS3–48c. His FECH mRNA was 71% of that of control 1 who has the genotype t/t.

The mild photosensitivity presented by the patients' father could be attributed to the increased erythrocyte protoporphyrin concentration, a phenomenon which was shown in one of our previous studies (Cohen et al 2005). However, at the present time, the cause(s) of increased protoporphyrin concentration in this particular individual is not fully understood. The *FECH* gene, or IVS3–48c in that sense, is likely to play a role in this setting. Although homozygous c/c alone might not be sufficient, additional factors, genetic and environmental alike, might act together to give rise to the clinical and biochemical abnormalities in this individual.

This study has shown that a combination of a disabling *FECH* mutation with the hypomorphic *FECH* IVS3–48c was the cause of EPP in two members of the Jewish family. The 8% prevalence of IVS3–48c in the Ashkenazi Jewish population is similar to that found in the European populations (Table 3). More importantly, this study presented the first case of an individual with c/c genotype who had both clinical and biochemical indications of EPP. These observations suggest that IVS3–48c is a functional variant of ferrochelatase and thus plays a role in the pathogenesis of EPP.

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