Original Article

A Novel Mutation in the *FECH* Gene in a Czech Family with Erythropoietic Protoporphyria and a Population Study of IVS3-48C Variant Contributing to the Disease

(hepatoerythropoietic porphyria / HEP / uroporphyrinogen decarboxylase / UROD / skin photosensitivity / red urine)

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Abstract. Erythropoietic protoporphyria (EPP), a chronic erythropoietic porphyria, is characterized by excess accumulation of protoporphyrin, particularly in erythroid cells. EPP inheritance is complex, almost always associated with two molecular defects. In most EPP patients, clinical expression requires coinheritance of a private ferrochelatase (*FECH*) mutation trans- to a hypomorphic FECH*IVS3-48C allele. This leads to a decrease of FECH activity below the critical threshold. This is characterized by cutaneous photosensitivity in early childhood such as itching, burning, swelling and redness in sun-exposed areas. Hepatic failure occurs in some patients (about 1–10 % of EPP patients), which may necessitate liver transplantation. We investigated a Czech family with two patients with manifested EPP in four generations. We found a novel mutation, c.84G >A, in the *FECH* gene in four individuals including proband and his mother (G84A transition in exon 2; p.W28*). Both clinically manifested probands inherited the hypomorphic IVS3-48C allele as well, while two clinically latent individuals with *FECH* mutation did not. To address the question whether the relatively low incidence of EPP in the Czech Republic might be due to lower frequency of the IVS3-48C allele, we screened for the frequency of the low expression allele in a control Czech (West Slaves) Caucasian population. Such study has not been performed in any Slavic population. Among 312 control individuals, there were no IVS3-48C/C (c.68-23C-T) homozygotes; 35 IVS3-48C/T heterozygous individuals were detected. The frequency of IVS3-48C allele was thus found to be 5.5 % in the Czech population, comparable to most West Caucasian populations.

Introduction

Erythropoietic protoporphyria (EPP; OMIM 177000) is caused by a decrease of the enzymatic activity of ferrochelatase, the eighth and the terminal enzyme of the haem biosynthetic pathway (protohaem ferro-lyase; EC 4.99.1.1) (Magnus et al., 1961). Mammalian FECH is a mitochondrial metalloenzyme with a [2Fe-2S] cluster as a cofactor. It catalyses the addition of the ferrous iron into protoporphyrin IX to produce haem, as shown in Fig. 1 (Dailey et al., 1994; Ferreira et al., 1994; Todd, 1994; Day et al., 1998; Anderson et al., 2001; Bloomer et al., 2006). The decrease in the levels of FECH in the bone marrow erythroid cells leads to the accumulation of protoporphyrin IX in the erythrocytes, plasma, skin, bile and faeces, leading to a clinical condition called EPP. EPP is a rare disease, but it is the most common type of porphyria that occurs in children (Sassa, 2006). In order for the symptoms of EPP to occur, the FECH activity should decrease to less than 30 % of normal (Kong et al., 2008).

EPP is characterized by cutaneous photosensitivity in early childhood such as itching, burning, swelling, bullae, vesicles and redness in areas exposed to sunlight

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Abbreviations: EPP – erythropoietic protoporphyria, FECH – ferrochelatase, PP – protoporphyrin.

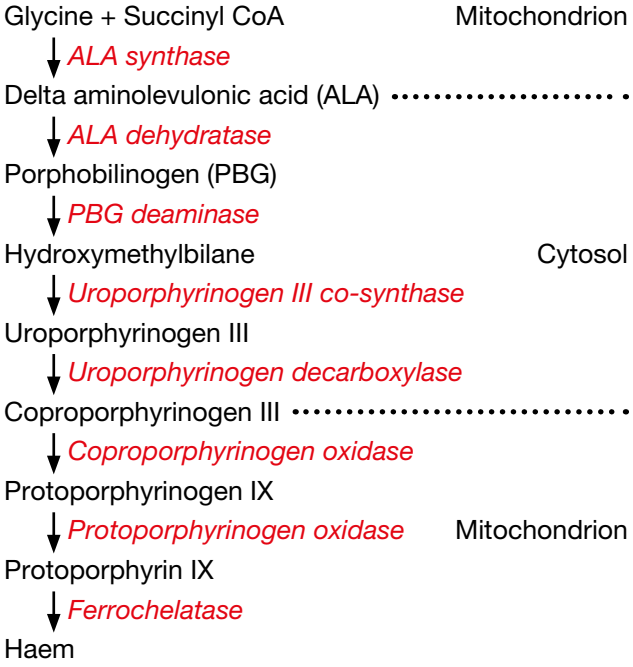

Fig. 1. Schematic representation of steps in the haem biosynthetic pathway

(Doss and Frank, 1989; Nordmann and Deybach, 1990; Meerman, 2000). Hepatobiliary disease results from an increase in hepatobiliary protoporphyrin excretion, which damages the cholangiocytes by oxidative stress and is responsible for the cholestatic phenomena (Bruguera et al., 2005; Holme and Herrero, 2007; Lyoumi et al., 2007; Lecha et al., 2009). Hepatic failure occurs in some patients (about 1–10 % of EPP patients), which may necessitate liver transplantation (Bloomer, 1988). The genetic cause of EPP was found to be due to mutations in the *FECH* gene, which is situated on chromosome 18q21.3 and contains 11 exons, spans over 45 kb of genomic DNA and has an open reading frame of 1269 bp. The gene encodes a polypeptide precursor of 423 amino acid residues, which later undergoes proteolysis to the mature protein that consists of 369 amino acids (Nakahashi et al., 1990). EPP can be inherited both as an autosomal dominant with low clinical penetrance or rarely as an autosomal recessive trait (Rüfenacht et al., 1998).

It has been previously reported that the EPP clinical expression requires two molecular defects: (a) most commonly, mutation in the ferrochelatase gene with the coinheritance trans- to a hypomorphic FECH*IVS3-48C allele (Gouya et al., 1999; Meerman, 2000; Richard et al., 2008; Tahara et al., 2010); (b) two loss-of-function mutations in the *FECH* gene or a gain-of-function mutation in 5'-aminolevulinate synthase 2 (*ALAS2*) leading to X-linked dominant erythropoietic protoporphyria (XLP) (Balwani et al., 2013).

It has been reported that the frequency of the IVS3-48C allele dramatically differs in various populations, being 45 % for Japanese population (Nakano et al., 2006) and < 1 % in black West African population, respectively (Gouya et al., 2006).

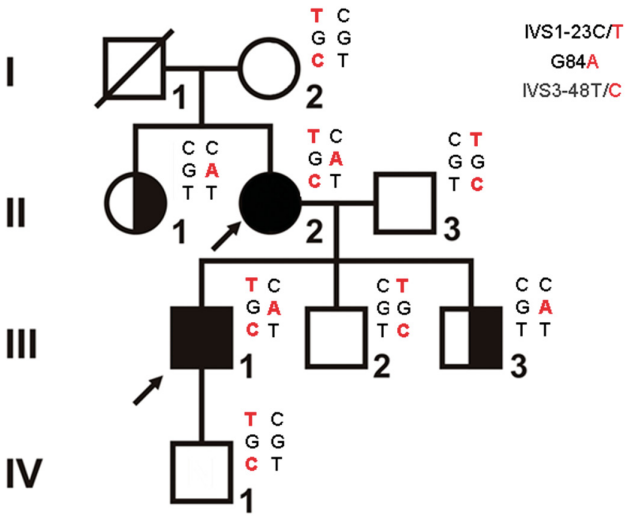

Fig. 2. Genealogical tree of the EPP family with G84A (p.W28*) mutation.
Polymorphisms are shown as haplotypes (left and right columns) with the mutant alleles in red, from top to bottom: IVS1-23C/T, mutation within *FECH* exon 2 G84A m; and the low expression allele polymorphism IVS3-48C>T. The pedigree of the proband's family: ○ – female, ■ – male, fully filled – clinically manifest porphyria, half-filled – clinically silent.

In this study, we investigated the molecular defect in the ferrochelatase gene in a mother and a son of Czech origin with manifested EPP and six other members of the family in four generations. We found a novel mutation, G84A, in both probands and we also studied the prevalence of IVS3-48C polymorphism required for the clinical manifestation of the disease not only in the family with EPP, but also in a control population of 312 Czech Caucasians (West Slaves). Such a study has not been performed yet in any Slavic population.

Material and Methods

Patients

We studied a Czech family of eight members in four generations, as shown in the pedigree in Fig. 2. The proband (40-year-old male) and his mother have suffered from skin photosensitivity since early childhood. The case of the proband was preliminarily reported earlier (Sperl et al., 2009). They were diagnosed as EPP patients based on clinical findings and biochemical investigations. The male patient suffered from liver failure and recently underwent liver transplantion, as described for EPP patients previously (Avnish Kumar et al., 2007; Richard et al., 2008; Casanova-Gonzalez et al., 2010; Dowman et al., 2011). Blood samples were obtained from all adult members of the family after receiving the informed consent. A blood sample of a child member of the family, IV/1, was obtained after informed consent from the parents. A total of 312 DNA samples of adults were acquired during the longitudinal collection

of control samples of healthy individuals from a Czech Slavic population in our laboratory. The population cohort of Czech controls consists of 163 males and 149 females of random ages from 18-year-old. The ethics committee of the First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague approved the study under No. 112/12.

Measurement of protoporphyrin in erythrocytes

Both probands were diagnosed as EPP patients based on the clinical findings, skin symptomatology, a distinct peak in the plasma emission scan at 635 nm and a high erythrocyte protoporphyrin content. Total erythrocyte porphyrins were measured according to Piomelli and Blake (Piomelli, 1977; Blake et al., 1992). Porphyrin determination is based on the separation of porphyrins from haem and spectrophotometry.

Genomic DNA analysis

Peripheral EDTA blood samples were obtained after informed consent from all family members. Total genomic DNA was isolated from peripheral blood leukocytes using a QIAamp DNA minikit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The 11 exons and the flanking intronic regions of *FECH* gene were amplified by polymerase chain reaction (PCR) using the primers listed in Table 1. (According to the published *FECH* sequence No AJ250235. http://www.ensembl.org/index.html; NG_008175 http://www.ncbi.nlm.nih.gov/) PCR amplification of the coding sequence and exon/intron borders of the *FECH* gene was performed by the primer pairs under optimized conditions. The PCR products were analysed by direct sequencing in an ABI prism 310 genetic analyser (Applied Biosystems, Waltham, MA). PCR products, polymorphisms IVS1-23 C > T and IVS3-48 T > C of the *FECH* gene were analysed in all the family members. The mutation was confirmed by re-sequencing the sense and antisense amplified fragments.

Population study

We screened the frequency of splice site modulator IVS3-48C, which is responsible for the occurrence of EPP in 312 controls of the Czech origin; 163 were females and 149 were males.

Results

Protoporphyrin measurements in erythrocytes

We measured the free protoporphyrin content in both probands with EPP (individuals II/2 and III/1 in Fig. 2) as well as in the clinically normal grandson (individual IV/1 in Fig. 2), finding it high in the probands (common in patients with EPP), while normal protoporphyrin concentrations were found in the grandson as shown in Table 2.

Genomic DNA analysis

Molecular genetic analyses of the ferrochelatase gene revealed a novel heterozygous transition, G84A, in exon 2, as shown in Fig. 3. The point mutation leads to a tryp-

Table 1. Primer pairs used for amplification of the corresponding 11 exons of the ferrochelatase gene

DNA				
*Exon 1:	sense	5'	TAGGAGTCCAGCAGGTTTTG	3'
	anti-sense	5'	GTGACAATAACCAAGGCTCT	3'
*Exon 2:	sense	5'	GTCAGGAATTATGCTCTGAGG	3'
	anti-sense	5'	AGCTATTGAAAGGAAGCCAAG	3'
*Exon 3:	sense	5'	AGATTAGAGTTTGCTGGCTG	3'
	anti-sense	5'	ACCATTACCAGATACGCATT	3'
*Exon 4:	sense	5'	TCTCTGCATGGGTGTTGTGT	3'
	anti-sense	5'	AAGGCTAAAGGTCAAGGGATAA	3'
*Exon 5:	sense	5'	GTCAGTGCCATAGGAAATTACA	3'
	anti-sense	5'	GACTGACCTGAACTCTCGTGT	3'
*Exon 6:	sense	5'	CACTAGAACTGACATCAATAATC	3'
	anti-sense	5'	AGTAAGGCTCAGAAGGACA	3'
*Exon 7:	sense	5'	CAATGCTGAGAGGCTGGACTGT	3'
	anti-sense	5'	CTTGCACTGGGCTTAGGACATA	3'
*Exons 8 and 9:	sense	5'	TCATCATTGGTGCAGGAGAC cc	3'
	anti-sense	5'	TGAGGACACCGTACATGCAA	3'
*Exon 10:	sense	5'	GCGAACAGTTGAAGTCAGAC	3'
	anti-sense	5'	CAGACATAGTTATAGGTGGGT	3'
*Exon 11:	sense	5'	CCAAGCCAGAGCGCTGACCT	3'
	anti-sense	5'	CTCTCCGTACCCTTTCGGGAGG	3'

Table 2. Protoporphyrin (PP) content in erythrocytes (normal values: total PP ≤ 2.00 µmol/µl)

Subject	Total PP (µmol/µl)	Zinc PP (%)	Free PP (%)
Proband I (son)	24.50	11	89
Proband II (mother)	23.64	7	93
Grandson (IV/1)	1.16	-	-

tophan to stop codon substitution (p.W28*). The amino acid tryptophan at position 28 is located in the area of mitochondrial targeting sequence spanning amino acid residues 1–62, which is removed during proteolytic processing, as shown in Fig. 4.

Mutation analyses were carried out in eight members of the proband's family. We found the same mutation in both probands (the mother and the son). Two other family members also showed the G84A mutation but are meanwhile clinically silent, as shown in the pedigree in Fig. 2.

The mother and the son with EPP are both heterozygous for intronic single-nucleotide polymorphisms (SNP) IVS3-48C/T, which is required for the expression of EPP, and IVS1-23 T/C with a presumed role in EPP pathogenesis (Gouya et al., 1999). Given the available data, it seems that the *CAT* haplotype (IVS1-23 T/C; G84A; IVS3-48C/T) combined with *TGC* haplotype precipitated the EPP manifestation in the two probands, whereas the combination of *TGC* or *CAT* with the wild-type *CGT* did not lead to any clinical consequences.

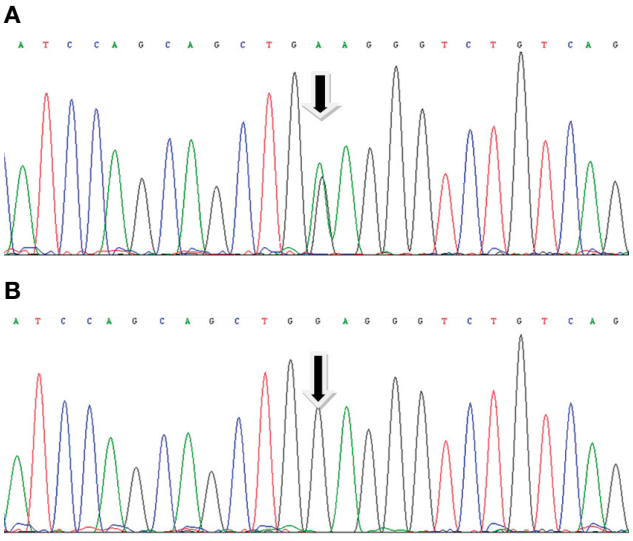

Fig. 3. **A**/ The nucleotide sequence of the human *FECH* gene shows the heterozygous G84A mutation in exon 2. **B**/ Wild-type sequence

Discussion

To date, more than 130 mutations in the *FECH* gene have been reported worldwide. We identified a novel missense mutation in the *FECH* gene in four members in a Czech family, a transition of G84A in exon 2 leading to a tryptophan to stop codon substitution causing premature ending of translation. For clinical manifestation of EPP, a synergy of a private mutation within the *FECH* gene and the presence of low expression IVS3-48C allele in trans is needed in the majority of cases (Gouya et al.,1999; Meerman, 2000; Richard et al., 2008; Tahara et al., 2010). Indeed, the two patients with manifest EPP have also inherited, apart from the G84A variant, the hypomorphic allele IVS-48C (and the IVS1-23T intronic variant), which is required for the EPP phenotype (Tahara et al., 2010). As both intronic variants are apparently inherited in a single haplotype block (Fig. 2), it is impossible to assess the distinct impact of IVS1-23T in combination with the G84A mutation. So far, just a few patients with EPP have been diagnosed in the Czech Republic despite good availability of a laboratory diagnosis of this disorder. According to our knowledge, only five families were diagnosed with EPP in the last three decades (Martasek P., personal observation).

We therefore performed a screening for the frequency of the low expression allele in a control Czech (West Slavic) Caucasian population. Such a study has not been performed yet in any Slavic population. Previously, it has been reported that the frequency of the IVS3-48C allele in the Japanese population was 45 % (Nakano et al., 2006), in Chinese (Han) 41 % (Kong et al., 2008), in Southeast Asian 31 %, in French Caucasians 6.4 % (Gouya et al., 2006), in British 13 % (Berroeta et al., 2007), in Ashkenazi Jews 8 % (Schneider-Yin et al., 2008), in Swiss population 7 % (Schneider-Yin et al., 2009), in Spanish 5 % (Herrero et al., 2007), in North African 2.7 %, in black West African populations < 1% (Gouya et al., 2006) and in Italians 1 % (Aurizi et al., 2007).

We screened the frequency of the IVS3-48C allele in 312 Czech control individuals; 149 subjects were males and 163 subjects were females. In the Czech control subjects we identified 277 IVS3-48T/T homozygotes and 35 IVS3-48C/T heterozygotes. Therefore, the frequency of the IVS-48C allele was estimated as 5.5 % in the Czech population, 5 % among males and 6 % among females, as shown in Table 3.

The healthcare professionals in the Czech Republic should be aware of the diagnosis of EPP and investigate patients with intolerance to sun exposure for the diagnosis of the disease. Nevertheless, we highlight the impor-

Fig. 4. The sequence of the first 62 amino acids of human ferrochelatase (UniProt accession number: P22830, http://www.uniprot.org) with an indication of the mutation p.W28* site in *FECH* leading to premature end of translation

Table 3. Incidence of distinct genotypes of the FECH IVS3-48T>G polymorphisms in males, females and total Czech controls

Genotype	Total male controls screened (N = 149)	Total female control screened (N = 163)	Total Czech controls screened (N = 312)
IVS-48T/C	15 subjects (5 % of total screened males carrying the C allele)	20 subjects (6 % of total screened females carrying the C allele)	35 subjects (5.5 % of total screened individuals carrying the C allele)
IVS-48T/T	134 subjects (90 % of total screened males)	143 subjects (88 % of total screened females)	277 subjects (89 % of total screened individuals)
IVS-48C/C	0	0	0

tance of the molecular analysis in the diagnosis of EPP, as it gives a full picture about the pathology for better understanding of the disease and helps physicians to identify asymptomatic carriers and therefore avoid further propagation of the disease.

Our results from the first Slavic Caucasian screening of 624 alleles in the Czech population thus indicate the overall IVS3-48C allele frequency of 5.5 %, comparable to the above-mentioned reports from other West Caucasian populations. While the frequency of the IVS3-48C allele is most likely not the reason for the low incidence of EPP in the Czech Republic, it remains to be determined whether a distinct protective variant or complex rearrangements of *FECH* or other genes involved in EPP pathogenesis underlie this phenomenon.

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