Novel Null-Allele Mutations and Genotype-Phenotype Correlation in Argentinean Patients with Erythropoietic Protoporphyria

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Erythropoietic protoporphyria (EPP) is an inherited disorder of porphyrin metabolism in which decreased activity of ferrochelatase (FECH) leads to accumulation of protoporphyrin IX (PP IX) in red blood cells, plasma, liver, and bile, and increased PP IX excretion in feces. Clinically, EPP is characterized by photosensitivity that begins in early childhood and includes burning, swelling, itching, and painful erythema in sun-exposed areas. Chronic liver disease is an important complication in a minority of EPP patients, and in some cases liver transplantation has been performed. So far, about 110 different mutations and several polymorphisms have been characterized in the human *FECH* gene. The relationship between mutations, polymorphisms, and porphyria development in Argentinean patients was investigated. This is the first genetic study carried out in the Argentinean population. In five Argentinean EPP families we detected three novel mutations: a deletion (451delT) producing a stop codon located 18 codons downstream from the mutation and two splicing mutations: IVS1-2A>G leading to exon 2 skipping and IVS4-2A>G, which causes the loss of the first 48 bp of exon 5. We also found two previously described mutations: C343T and 400delA, which produce stop codons. All patients had an FECH activity 25% of normal and also had the polymorphisms –251A>G in the promoter region and IVS1-23 C>T and IVS3-48 T>C. Our findings provide supporting evidence for the concept that the inheritance of the low expression allele IVS3-48C *in trans* with a mutation in the *FECH* gene is necessary for EPP to become clinically manifest.

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INTRODUCTION

Erythropoietic protoporphyria (EPP, MIM 17.700) is an inherited disorder of porphyrin metabolism first described by Magnus *et al*. (1). EPP is due to decreased activity of ferrochelatase (FECH) (E.C.4.99.1.1), the enzyme that catalyses the chelation of iron into protoporphyrin IX (PP IX) to form heme. The deficient activity leads to the accumulation of PP IX in red blood cells, plasma, liver, and bile, and an increased PP IX excretion in the feces (Figure 1).

Clinically, EPP is characterized by photosensitivity that begins in early childhood and includes burning, swelling, itching, and painful erythema in sun-exposed areas. Chronic liver disease, which occurs in up to 30% of EPP patients, can become an important complication in a minority of patients (2–4). In a limited number of cases, liver transplantation has been performed (2,3).

The human *FECH* gene was cloned, sequenced, and mapped to the long arm of chromosome 18 (5,6). The gene contains a total of 11 exons and spans about 45 kb of genomic DNA. The cDNA has an open reading frame of 1269 bp encoding a protein of 423 amino acid residues. The mature protein consists of 369 amino acids (7).

So far, more than 110 different mutations have been characterized in the human *FECH* gene, of which about 70% are null allele mutations (Human Gene Mutation Database: http://www.hgmd.org/). In addition, several polymorphisms have also been detected (http://www.ncbi.nlm.nih.gov/SNP).

In most cases, only one gene allele is affected by an inactivating mutation. EPP is transmitted as an autosomal dominant trait but with a low clinical penetrance because most mutation carriers remain asymptomatic despite having an FECH activity of about 50% of normal. In symptomatic individuals with EPP the

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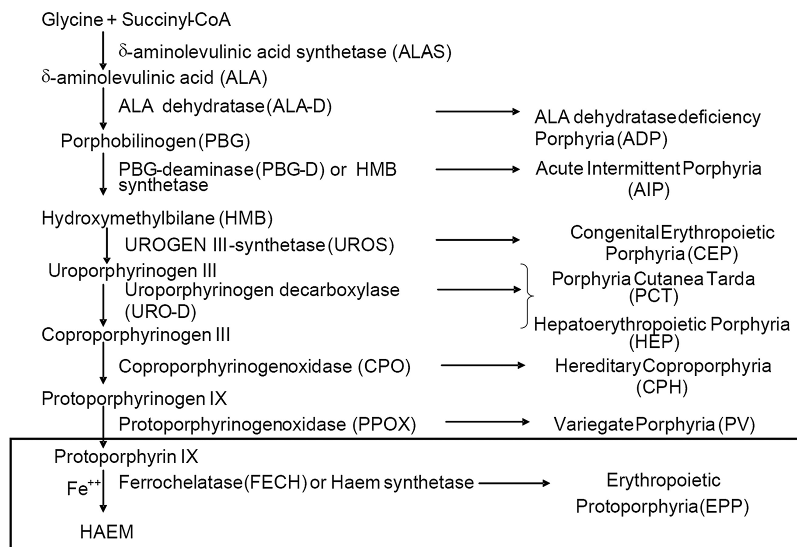

Figure 1. Heme pathway: enzymes involved and associated porphyrias.

activity of FECH is in general lower than the 50% that would be expected from an autosomal dominant inherited disease. Brenner's hypothesis (8) that the mutated *FECH* allele could have a dominant negative effect would explain the reduced enzyme activity observed in patients, but it does not explain the incomplete penetrance found in EPP families. In 1996 Gouya *et al*. (9) showed that the EPP phenotype in an affected family was the result of the coinheritance of a normal allele of low expression (about 50%) and a mutant allele. Later, it was proposed that this low-expression allele, which coinherits with a mutated allele resulting in an overt EPP, is strongly associated with the haplotype –251G: IVS1-23T: IVS2µsatA9, which was present in about 6.5–11.5% of a control group (10). More recently, it was established that the mechanism responsible for the low-expression FECH was the IVS3-48T>C transition that modulates the use of a constitutive aberrant splice site, 63 bp upstream of the normal site. Thus, the presence of a T in the position IVS3-48 was shown to produce about 20% aberrantly spliced mRNA, whereas the presence of a C in this position leads to a higher presence, about 40%, of the abnormal mRNA, which would be easily degraded by the so-called nonsense-mediated mRNA decay, producing a lower steady-state level of *FECH* mRNA (11,12). Although the inheritance of an IVS3-48C allele trans to the mutation explained the phenotypic expression in most families (4,12–14), autosomal recessive inheritance can cause overt EPP in patients who do not have the low-

Table 1. Biochemical data of EPP probands and relatives.*

Subject	Age, years	Blood porphyrins, µg/100 mL RBC	Fecal porphyrins, µg/g dry weight	Plasma protoporphyrin index (λ nm)	FECH activity, pmol/mg protein	PP IX, µmol/L RBC
Family 1						
Proband	48	1005	375	6.28 (630)	160	61
Brother	57	80	53	1.30 (618)	785	0.9
Sister	59	92	65	1.30 (618)	707	1.8
Family 2						
Proband	43	661	235	1.86 (630)	178	35
Daugther	15	120	98	1.30 (618)	ND	ND
Daugther	10	107	ND	1.28 (618)	ND	ND
Patient 3						
Proband	35	1206	1240	2.90 (630)	126	52
Patient 4						
Proband	26	614	164	4.50 (630)	174	33
Family 5						
Proband	21	870	645	3.60 (630)	131	33
Brother	15	550	656	2.25 (630)	177	19
Sister	18	142	112	1.30 (618)	339	1.4
Mother	47	116	114	1.30 (618)	750	6.0
Father	49	175	ND	1.30 (618)	ND	ND
Normal value		<190	<130	<1.30 (618)	355–750	<1.5

*Clinical and biochemical symptoms at the age of diagnosis for Argentinean EPP patients and the available relatives studied at the molecular level are shown. Methodology was as described in Materials and Methods. ND, no data, because samples were unavailable or limited.

expression allele. A small number of cases of recessive inheritance have been reported (15,16).

To date, EPP has been diagnosed in 41 individuals from 35 families in our Research Centre on Porphyrins and Porphyrias in Argentina. This study is the first to investigate EPP at the molecular level in affected individuals in Argentinean unrelated families. We looked for the *FECH* gene mutation as well as three of the reported polymorphisms associated with the low-expression allele. We found three novel null mutations, two splicing defects and one small deletion, and two previously reported mutations. All patients were heterozygous carriers of EPP, and all symptomatic individuals in each family also inherited the IVS3-48C allele strongly associated with the low-expression *FECH* allele. These results in Argentinean EPP patients provide support for the proposed mechanism for the phenotypic expression of EPP and reinforce the idea that this phenomenon is a general occurrence for this porphyria.

Table 2. Primers for PCR reaction.

DNA		
Promotor	Sense	5′ ctt gta ttg tga cta ggc ag 3′
	Antisense	5′ cca gca gtg gcc gag ccg gg 3′
Exon 1	Sense	5′ cgg aca cct ggg cgc gcc gcc 3′
	Antisense	5′ cct ggc cct ggc ggc cgc cg 3′
Exon 2	Sense	5′ cct ctt cag aag aaa tta c 3′
	Antisense	5′ ggt gtc tgc atc gat aaa gag 3′
Exon 3	Sense	5′ aag tgt gac aaa tca acc g 3′
	Antisense	5′ ttg aga gac aca cat gtc aat g 3′
Exon 4	Sense	5′ cgt ttc tca ggt tgc taa gc 3′
	Antisense	5′ att tca taa cta ctt cga aag 3′
Exon 5	Sense	5′ ctc tga gga atc tat ata agg 3′
	Antisense	5′ tt ggt tat ttt tgc cag ca 3′
Exon 6	Sense	5′ gag tct gtt ttt att tca cc 3′
	Antisense	5′ atg aga agc tga ttc aca cta g 3′
Exon 7	Sense	5′ cct tcc att tct ttc ttc cc 3′
	Antisense	5′ gaa aat gaa atc acc caa tcc 3′
Exon 8	Sense	5′ cag agg tct cgg ggc ctc cc 3′
	Antisense	5′ ttt acc aat aag agc tgg cc 3′
Exon 9	Sense	5′ ggg gta cta cag acc gtc ag 3′
	Antisense	5′ gat gga aaa agg cag atg gg 3′
Exon 10	Sense	5′ gga aaa taa aat ata tct gtg 3′
	Antisense	5′ gag ttt ctc aga gga tta ctc 3′
Exon 11	Sense	5′ ggc atg gca gac gag cag acc c 3′
	Antisense	5′ cac aat ttg tac cca aag gc 3′
cDNA		
RT primer		5′ gag gtt ggg cat ttg cct aac g 3′
Promotor/Exon 1	Sense	5′ ccc agg caa tgc gtt cac tc 3′
Exon 9	Antisense	5′ ttc gtc tgt ttg agg acc ca 3′

MATERIALS AND METHODS

Biochemical Studies

Peripheral blood samples were collected in heparinized test tubes from the patients and their relatives.

Total porphyrins in whole blood samples and feces and plasma porphyrins (plasma porphyrin index) were measured by methods previously described (17). Ferrochelatase was measured with a modified method that was a combination of those described by Li and Lim (18) and Rossi *et al*. (19), and erythrocyte protoporphyrin was quantified by the modified method of Bailey and Needman (20). Biochemical data are shown in Table 1.

Mutational Analysis

Genomic DNA was isolated from peripheral EDTA blood samples by use of the InstaGene Whole Blood Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Total RNA was isolated from Epstein-Barr virus transfected lymphoblastoid cell lines (21) and complementary DNA was obtained with reverse transcriptase.

The 11 exons of *FECH*, including the flanking intronic regions, were amplified by polymerase chain reaction (PCR) with the primers listed in Table 2 (according to the published *FECH* sequence no. AJ250235). Sequencing was performed using a DNA sequencing kit (Applied Biosystems, Foster City, CA, USA), and reactions were run on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Waltham, MA, USA). Amplification of cDNA samples were performed with primers listed in Table 2, and PCR products were sequenced with internal primers.

Polymorphisms –251 A>G in the promoter region and IVSI-23 C>T and IVS3-48 T>C of the *FECH* gene were also studied in all the patients and their available relatives.

Patients

Five unrelated Argentinean EPP families were studied. Informed consent was obtained from all patients prior to their inclusion in the study.

Family 1. The proband is a 48-year-old woman with EPP diagnosed when she was 35 years old. She had cutaneous photosensitivity since she was 6 months old. No other family members have had any clinical symptoms of EPP. Samples from her sister (59 years old) and her brother (57 years old) were also available.

Family 2. The proband is a 43-year-old man with EPP diagnosed when he was 26 years old. He had mild photosensitivity that started when he was 4 years old. His father's brother also has EPP, which was diagnosed when he was 34 years old. Samples from the proband's two daughters (15 and 10 years old) were also available for investigation.

Patient 3. The proband is a 35-year-old man with EPP diagnosed when he was

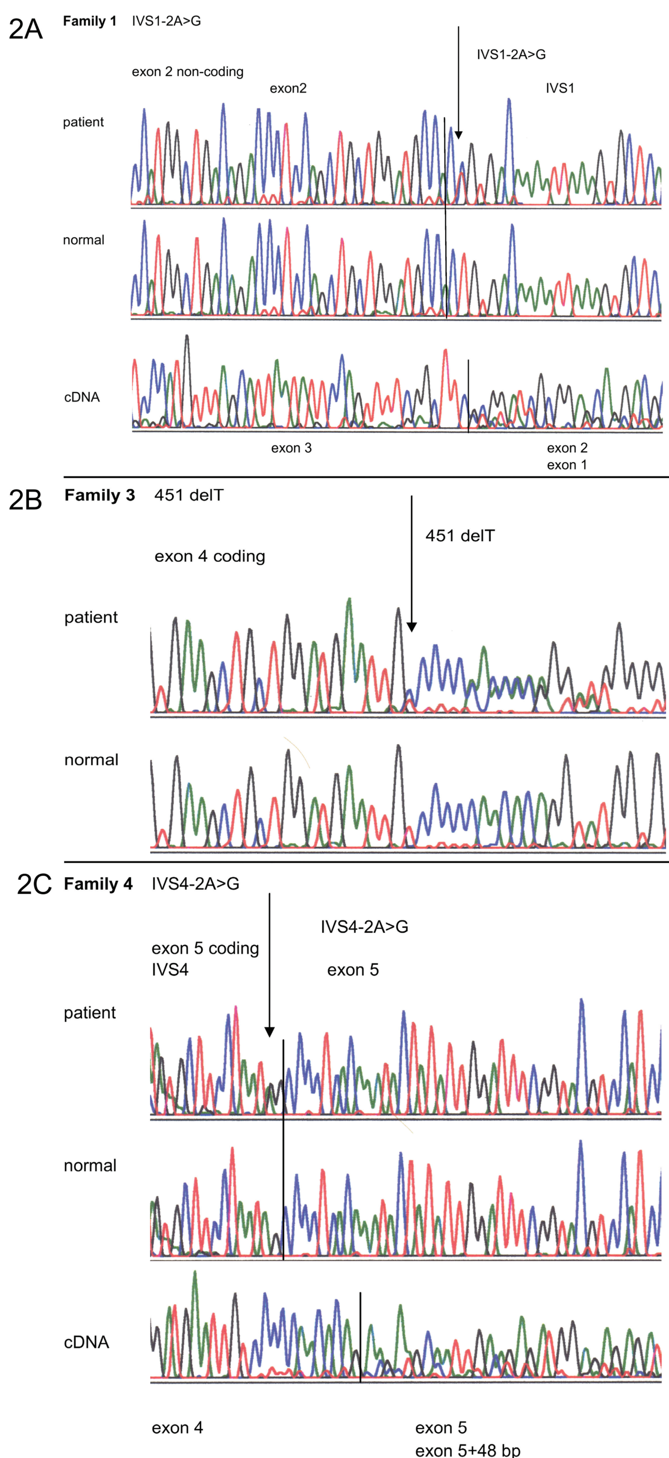

Figure 2. Sequence results of novel mutations found in three families. (A) Family 1: mutation IVS1-2A>G (arrow). The noncoding genomic sequences of patients and controls are shown. Skipping of exon 2 is seen in the patient's cDNA. (B) Family 3: mutation 451delT (arrow). The coding genomic sequences of patients and controls are shown. (C) Family 4: mutation IVS4-2A>G (arrow) close to the intron 4/exon 5 boundary (line). The coding genomic sequences of a patient and a control subject are shown. Loss of the first 48 bp in exon 5 can be seen in the patient's cDNA.

29 years old. This patient has had cutaneous manifestations since he was 5 years old. One nephew shows similar cutaneous symptoms. There were no relatives available for testing.

Patient 4. The proband is a 26-year-old man with EPP diagnosed when he was 15 years old. In this patient cutaneous photosensitivity began at the age of 9 years, after he had received antihistamines for an infectious syndrome. His parents and his three sisters show no clinical symptoms and they are also biochemically normal, but no samples were available for genetic studies.

Family 5. In this family two brothers exhibit clinical symptoms of EPP. One of them is 21 years old; he first showed cutaneous signs at the age of 6 years and his EPP was diagnosed 1 year later. The other patient is his 15-year-old brother, who has EPP that was diagnosed when he was 2 years old and showed skin photosensitivity. Samples from their mother (47 years old), their father (49 years old), and their sister (18 years old) were also available for testing. Neither their parents nor their sister have any clinical or biochemical manifestations of EPP.

RESULTS

All symptomatic patients had increased values of PP IX in red blood cells and feces and a value of FECH activity about 25% of the control. It is of note that the value of 6.0 given for the mother in family 5 is not significant even though it is out of the range of normal values (Table 1).

In family 1, sequencing of amplified genomic DNA with both sense and antisense primers showed a novel point mutation in the acceptor site of splicing in intron 1 (IVS1-2 A>G). To analyze the effect of this mutation in the mRNA, we performed processing, amplification, and sequencing of cDNA, which showed skipping of exon 2 in this patient (Figure 2A). Neither her sister nor her brother had the mutation. The proband inherited haplotype –251G/G; IVS1-23 T/T; IVS3-48 T/C for the polymorphisms studied (Table 3).

In family 2, we found a known frameshift mutation in exon 4, 400delA,

Table 3. Mutations and polymorphisms in the *FECH* gene

Subject	Symptomatology	Mutation	Promotor region –251A>G	IVS1-23C>T	IVS3-48 T>C
Family 1					
Proband	Yes	IVS1-2 A>G	G/G	T/T	T/C
Brother	No	Normal	A/A	C/C	T/T
Sister	No	Normal	A/G	C/T	T/C
Family 2					
Proband	Yes	400delA	A/G	C/T	T/C
Daugther	No	400delA	ND*	C/C	ND
Daugther	No	400delA	ND	ND	ND
Patient 3					
Proband	Yes	451delT	A/G	C/T	T/C
Patient 4					
Proband	Yes	IVS4-2 A>G	A/G	C/T	T/C
Family 5					
Proband	Yes	343 C>T	A/G	C/T	T/C
Brother	Yes	343 C>T	A/G	C/T	T/C
Sister	No	343 C>T	A/A	C/C	T/T
Mother	No	Normal	A/G	C/T	T/C
Father	No	343 C>T	ND	ND	ND

*ND, no data because samples were unavailable or limited.

which produces a stop codon at codon position 144 as described by Wang *et al*. in 1997 (22). The proband's two daughters are carriers for this mutation. The patient presented the –251A/G; IVS1-23 C/T; IVS3-48 T/C haplotype, and one of his daughters inherited the normal C/C allele for the IVS1-23 polymorphism, but unfortunately we could not analyze the IVS3-48 T/C polymorphism for either of his daughters (Table 3).

In patient 3, a novel small deletion in exon 4 was detected, 451delT, which produces a frameshift and a stop codon at codon position 169 (Figure 2B). The patient also presented the –251 A/G; IVS1-23 C/T; IVS3-48 T/C polymorphisms (Table 3). No relatives were available for the study.

In patient 4, another novel point mutation in the acceptor site of splicing of intron 4 was detected, IVS4-2 A>G. Sequencing of the cDNA resulted in the loss of the first 48 bp of exon 5 but not the skipping of the entire exon, which is probably attributable to the use of the first AG downstream of the mutation site as a cryptic splicing site (Figure 2C). The proband has the haplotype: –251A/G; IVS1-23 C/T, IVS3-48 T/C (Table 3). No relatives were available for the study.

In family 5, both brothers showed a known nonsense mutation in exon 4, 343C/T, yielding a stop codon at the arginine 115 (R115X), as described by Henriksson *et al*. in 1996 (23). Their mother was normal, but both their father and sister had the same mutation. The mother had the low IVS3-48C–expressed *FECH* allele. Both patients have inherited the haplotype –251 A/G; IVS1-23 C/T; IVS3-48 T/C, whereas their asymptomatic sister inherited the normal haplotype –251A/A; IVS1-23 C/C; IVS3-48 T/T (Table 3).

DISCUSSION

The worldwide prevalence of EPP has been estimated to be 1:75,000 to 1:200,000 inhabitants. In the last 25 years EPP has been diagnosed by biochemical analysis in 41 patients from 35 unrelated Argentinean families, so the estimated prevalence of EPP in Argentina is about 1:800,000.

Mutations in the *FECH* gene causing EPP are highly heterogeneous, and most of them are unique to a family. To date more than 110 different disease-causing mutations have been detected in the *FECH* gene (Human Gene Mutation Database: http://www.hgmd.org/). Of these mutations, about 70% are "null allele mutations" as defined by Minder *et al*. (24).

The study we report is the first to include analysis at the molecular level of samples from members of five unrelated Argentinean EPP families. We have found three novel mutations and two mutations that have been previously described (22,23). The three new mutations were null mutations: two splicing mutations and one small deletion.

In the proband of family 1 a splicing mutation due to the transition a→g in intron 1 leading to exon 2 skipping (IVS1-2 A>G) was detected on the basis of DNA and cDNA sequencing results (Figure 1).

In patient 4 we detected another new splicing mutation in intron 4 (IVS4-2 A>G) (Figure 2C). This mutation alters the acceptor splice site of intron 4, producing the loss of the first 48 bp of exon 5 due to the use of the first cryptic site (AG) located downstream from the mutation (Figure 2C). This is the first report of the use of a cryptic site among all the mutations described for the *FECH* gene.

A new deletion (451delT) that produces a stop codon located 18 codons downstream from the deletion was found in patient 3 (Figure 2B). In the other two families, we detected two previously described mutations producing stop codons: 400delA (22) and 343C>T (23).

All six of the symptomatic patients had an FECH activity 25% of the normal value (Table 1) and the haplotype –251 A/G, IVS1–23 C/T, IVS3-48 T/C (Table 3), with exception of patient 1, who was homozygous for the first two. These findings are in agreement with the conclusion that the inheritance of a low-expressed allele together with the mutation in the *FECH* gene are necessary for the clinical expression of this porphyria (4,13,14,16,25–27).

In family 2, both daughters (17 and 12 years old at the time of this report) are carriers of the *FECH* mutation. Because the oldest daughter is still asympto-

matic and bears the normal allele for the IVS1-23 polymorphism, we may assume that she will have the normal haplotype for the other two polymorphisms as well, because it is likely that her sister also has the normal haplotype for the IVS3-48C low-expression allele. It is highly possible that both daughters will remain asymptomatic.

In family 5, the proband and his brother carried both the 343C>T mutation and the low-expression allele and were symptomatic. Their sister, who carried the mutation and had the normal haplotype and an FECH enzyme activity 50% of the normal value, was asymptomatic. On the basis of findings reported by Gouya *et al*. (10) she has a risk of less than 2% to become symptomatic. Moreover, because the patient and his brother have inherited the low-expression haplotype from their mother, we can assume that the asymptomatic father in this family must have the haplotype –251A/A, IVS1-23C/C, IVS3-48T/T, and thus these individuals did not manifest the disease. This mutation, first described in a Finnish patient (22), has been also found in patients from France (28), Spain (26,29), Italy (30), Sweden (13), and China (31). Of the seven patients who carried the 343C>T mutation, only three suffered from liver disease (13,28,31). The reason for this finding is not yet well understood.

Although it has been observed that null-allele mutations inducing the production of a truncated protein are generally associated with liver complications, our patients, all of whom carried null allele mutations in the *FECH* gene, had not shown any sign of liver disease up to the time of this report. We do however recommend that their liver function be periodically monitored to detect any complications.

We conclude that early knowledge of the mutation and associated polymorphisms in a biochemically diagnosed individual with EPP is important to make a proper diagnosis and to initiate symptomatic treatment as well as surveillance and timely interventions to avoid severe protoporphyrin-related liver disease.

Finally, these results in Argentinean EPP patients add further support to the observation that clinically overt EPP results from the coinheritance of a normal low-expression allele *in trans* to a mutated *FECH* allele, and that this seems to be a general phenomenon of EPP expression. Studies at a molecular level allow us not only the accurate diagnosis of asymptomatic carriers but also the identification of the factors that determine the prognosis of EPP and thus increased accuracy in genetic counseling of EPP families.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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