Allele variation in the ferrochelatase gene in a Chinese pedigree of erythropoietic protoporphyria

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Research Article

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Allele variation in the ferrochelatase gene in a Chinese pedigree of erythropoietic protoporphyria

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Abstract

Background: Erythropoietic protoporphyria (EPP) is characterized by photosensitive skin lesions and various liver injuries. EPP is an inherited disease closely related to single nucleotide polymorphisms (SNPs) in the ferrochelatase gene (*FECH*). We aim to analyze *FECH* mutations in a Chinese pedigree in order to help clarify the disease's pattern of inheritance.

Results: We genotyped the *FECH* gene in a male proband with EPP with liver injury and in his family members in Fujian, China. Since the *FECH* allele (IVS3-48C) has been associated with up to 98% of patients with EPP, we investigated this mutation in 200 unrelated controls in Fujian. The deletion shift mutation c.757_761delAGAAG was detected in the proband and his brother, father, two uncles, and grandmother. The proband, his brother and two uncles, all of whom showed photosensitive lesions, carried both the c.757_761delAGAAG allele and the IVS3-48C allele. We also detected the mutant allele IVS8-61_-62delCT in this pedigree. In the 200 unrelated subjects, 31.47% carried the IVS3-48C allele. In a pedigree of EPP, we detected the deletion shift mutation c.757_761delAGAAG, which is the first report of such a mutation in China.

Conclusions: Based on our data, the mode of inheritance requires a haplotype formed by two loci, IVS3-48T>Cand c.757_761delAGAAG.

Keywords: erythropoietic protoporphyria, ferrochelatase, liver injury, gene mutation study

1 Introduction

Incidence of erythropoietic protoporphyria (EPP) varies between 1 per 75 000 and 1 per 200 000 globally[1]. EPP is an inherited metabolic disease in which abnormalities in the *FECH* gene lead to reduced activity of the encoded enzyme, ferrochelatase. This enzyme facilitates the binding of ferrous iron to protoporphyrin IX during the production of hemoglobin. When the enzyme activity is reduced, excessive protoporphyrin IX accumulates in erythrocytes and other tissues, such as the skin and liver. Neonates with EPP may suffer cutaneous injuries upon their first sun exposure. In addition, persistent presence of protoporphyrin in the liver triggers bile accumulation and leads to liver and gallbladder disease due to oxidative stress. Approximately 5-20% of EPP patients show hepatic involvement[2]: 5-10% develop liver cirrhosis, while 5% of patients develop terminal liver disease, which can lead to death if appropriate treatment is not administered[3].

Preliminary diagnosis of EPP can be made based on the presence of photosensitivity, liver injury and nervous system symptoms in patients who also show significant increases in free protoporphyrin in erythrocytes, no detectable urinary protoporphyrin, and birefringent optical protoporphyrin crystals in hepatocytes and Kupffer cells in liver biopsies. However, *FECH* genotyping is considered the gold standard for diagnosing EPP.

Development of EPP has been relatively well studied in non-Chinese subjects. The most common mode of inheritance is incomplete penetrance of autosomal dominance, which is intimately associated with single nucleotide polymorphisms (SNPs). These studies suggest that symptomatic EPP requires the mutant allele and low-expression *FECH* alleles from both parents.[4]. The most common low-expression allele is IVS3-48C, in which the SNP lies in

intron 3; this allele is associated with EPP in up to 98% of non-Chinese patients [4]. The IVS3-48C intron can insert 63 bp upstream of the normal splice site, leading to abnormally spliced mRNA containing a premature stop codon, which markedly reduces expression of full-length enzyme.

Although some studies in China have found *FECH* mutations associated with EPP, whether the pattern of disease inheritance is similar to that in non-Chinese is unclear. The present study sought to identify novel mutations in a pedigree of EPP and examine its mode of inheritance in Chinese.

2 Materials and Methods

2.1 Case report

A 21-year old male resident of Hui'an, Fujian, China was admitted in 2014 to The 900th Hospital of the People's Liberation Army Joint Service Support Force due to bleeding of the upper gastrointestinal tract. The patient presented with yellow sclera, dark yellow urine, itching and burning sensation in the skin, and intense lumbar pain. The patient reported both a personal and family history (two uncles and one brother) of various skin injuries upon intense exposure to the sun. Physical examination revealed pigmentation in the skin of the neck and bilateral palms, hyperkeratinization, as well as open lesions and scars on the skin of both knees (Figure 1). The liver was palpable 2 cm below the rib cage and the spleen at 8 cm below. Laboratory examination showed the following abnormal indices: hemoglobin, 70 g/L (normal, >110 g/L); platelets, 43×10^9/L (>100×10^9 /L); gluten-propyl aminotransferase,342 U/L (< 40 U/L); gluten-oxalaminotransferase, 210 U/L (< 40 U/L); glutamyltransferase,146

U/L (< 40 U/L); total bilirubin, 94.5 μmol/L (< 25 μmol/L); and direct bilirubin, 51.4 μmol/L (< 7.5 μmol/L). Serum was positive for surface antibody against hepatitis B virus, but negative for anti-neutrophil cytoplasmic antibodies and for antibodies against hepatitis C virus, hepatitis E virus, nuclear antigen, and double-stranded DNA. Additionally, blood was negative for Epstein-Barr virus DNA and cytomegalovirus DNA, ceruloplasmin, α1-antitrypsin, and glucose-6-phosphate dehydrogenase. Ferritin, total iron binding capacity and serum iron were all within the normal range. Urine analysis was negative for urobilinogen, but positive for bilirubin. Upper abdominal computed tomography angiography revealed hepatic cirrhosis, splenomegaly with portal hypertension and esophageal varices (Figure 2). Liver biopsy showed hepatic fibrosis, intrahepatic bile thrombosis and lipofuscin deposition in hepatocytes. The tubular or granular liver tissue showing bright red birefringence under polarizing lenses, with the Maltesecross visible. (Figure 3). Based on clinical manifestations of the patient and family history of skin symptoms, EPP was considered the most likely diagnosis.

The patient underwent *FECH* gene testing at the Molecular Pathology Center, Affiliated Hospital of Air Force Aviation Medicine Institute. A deletion shift mutation in exon 7 (c.757_761delAGAAG) and a synonymous mutation in exon 9 (c.921A>G) were identified, and EPP was definitively diagnosed. The patient was told to avoid sunlight and a high carbohydrate diet, and was given intravenous infusion of 10% dextrose in addition to symptomatic treatment. The patient improved and was discharged from the hospital approximately two weeks later.

2.2 Extraction and analysis of genomic DNA

EDTA K2 tubes were used to collect 5 mL of peripheral blood from the proband and pedigree members. Genomic DNA was extracted using a whole blood genomic DNA extraction kit as instructed by the manufacturer (Omega, USA). DNA purity and concentration were measured using a ND-1000 spectrophotometer (Shanghai Spectrum, Shanghai, China).

The TriFast kit (Shanghai Chromysky Medical Research, Shanghai, China) was used to determined kinship among pedigree members. The kit examines 20 short tandem repeat SNP markers, including 8 on chromosome 21, 6 on chromosome 18, and 6 on chromosome 13.

The c.757_761delAGAAG (SNP ID: rs780942159) locus was amplified by PCR using FAM-labeled PCR primers, and the PCR products were subjected to capillary electrophoresis using the 3500Dx genetic analyzer (ABI, USA) (Table 1). Amplified products of IVS3-48T>C (SNP ID: rs2272783) and c.921A>G (SNP ID: rs536560) were genotyped using Sanger sequencing (Table 1). In addition, the locus IVS3-48T>C was genotyped in 200 unrelated subjects living in Fujian province after informed consent was obtained.

3 **Results**

Kinship was confirmed in 9 members of the pedigree by short tandem repeat SNP genotyping (Figure 4). In these 9 samples (Table 2), we observed a deletion mutation within the pedigree, c.757_761delAGAAG, and three SNPs: IVS3-48T>C, c.921A>G and IVS8-61_-62delCT (Figure 5). We found that the proband's grandmother, father, two uncles and brother were heterozygous for the deletion mutation c.757_761delAGAAG in exon 7. Comparative analysis of genotypes at six short tandem repeat loci on chromosome 18 showed that the proband's haplotype was inherited from the grandmother through the father.

Genotyping revealed that both the proband and his brother were heterozygous for IVS3-48T>C due to a mutation inherited from their mother. On the paternal side of the proband's pedigree, one aunt and two uncles were all heterozygous at this locus, while the grandmother was wild-type homozygous, suggesting that the variant allele was inherited from the proband's grandfather.

The proband and his father and mother were heterozygous for c.921A>G in exon 9, but his brother was homozygous for the mutation. This indicates that one of the mutant alleles forms a haplotype with the deletion allele, c.757_761delAGAAG, which was inherited from the proband's father and grandmother. His grandmother also passed on the haplotype to his two uncles.

Another mutant allele, IVS8-61_-62delCT (SNP ID: rs60626363), was detected when testing the amplicon of the c.921 A>G locus. The proband and his brother and father were heterozygous at this locus, while his mother was homozygous. Therefore, the deletion mutation allele came from the father. Considering that the c.757_761delAGAAG mutant

allele in the proband and his brother also came from the father, it appears that IVS8-61_-62delCT and the c.757_761delAGAAG deletion mutant alleles form a haplotype in the proband's father; in other words, the two loci are located on the same chromosome. This haplotype came from the proband's grandmother. This implies that the two uncles of the proband also inherited the haplotype from the grandmother, including the deletion allele IVS8-61_-62delCT. These considerations led us to construct a pedigree map tracing the haplotype formed by the three loci IVS3-48T>C, c.757_761delAGAAG and IVS8-61_-62delCT (Figure 4).

We also examined the IVS3-48T>C locus in 200 non-related subjects in Fujian, China. After excluding three subjects whose sequencing failed, 93 were genotyped as IVS3-48T/T, 84 as IVS3-48T/C, and 20 as IVS3-48C/C. Therefore, the mutant allele frequency was 31.47%.

4 Discussion

Though a preliminary diagnosis of EPP can be made based on several measures, gene testing allows definitive diagnosis. The current study provides the first report on deletion mutation c.757_761delAGAAG (rs780942159) in Chinese people, which we found in our EPP pedigree but not in 200 unrelated normal subjects. This type of deletion causes a frameshift and generates a premature stop codon R, producing a truncated protein consisting of 321 amino acids. The affected individual shows reduced ferrochelatase activity, resulting in EPP. The mutant locus was first reported in 173 French EPP patients [5], then later in an Italian EPP pedigree, but not in 50 unrelated normal subjects[6]. The mutation was identified in 12 patients in 18 South African EPP pedigrees [7]. We conclude that this mutant locus is pathogenic in Chinese and non-Chinese populations.

We also detected a heterozygous mutation in the IVS3−48T>C locus in the pedigree. Low expression of the IVS3-48C allele is associated with EPP[4]. The mRNA containing this allele is abnormally spliced and so suffers greater non-sense mediated decay, reducing FECH activity by 20%[4].

The IVS3-48T>C mutation appears to play a strong role in EPP, as one study detected the IVS3−48C allele in 98% of 173 EPP patients showing a dominant inheritance pattern[5]. We found a frequency of 31.47% for the IVS3−48T>C mutation among Chinese in Fujian without EPP. Studies across China, including Shanghai, Hong Kong, and Beijing, found mutation frequencies of 28-41.35% [8][9][10]. (One Chinese study failed to detect the IVS3-4C allele in 50 normal subjects from Xi'an, and they concluded that the allele frequency was <1%[11].) Several studies outside China have suggested a lower frequency of the mutant allele

in the normal population, including 11.3% in France, 2.7% in north Africa, <1% in west Africans,31.0% in southeast Asians[5],43% in Japanese[12]and10.5% in Spaniards[13].These results suggest that the frequency of the IVS3-48C allele is markedly higher in Chinese than in Caucasians and Africans.

In our study, four symptomatic EPP patients carried the c.757_761delAGAAG mutation and IVS3-48C mutant allele. Analysis showed that the c.757_761delAGAAG mutant gene came from the proband's grandmother in all four patients, while the IVS3-48C mutant allele in the proband and his brother came from the proband's mother. The IVS3-48C mutant allele in the two uncles of the proband came from his grandfather; although no sample from the grandfather was available, the proband's grandmother was homozygous for the IVS3-48T allele. Therefore, loss of function of the *FECH* gene occurred in both copies of chromosome 18 in the four patients. The frameshift mutation in *FECH* in one copy of chromosome 18 was due to c.757_761delAGAAG deletion, and the IVS3-48C allele in the other copy of chromosome 18 caused abnormal mRNA splicing. One possibility is that the most common mode of inheritance of EPP is incomplete penetrance of autosomal dominance, which does not completely follow Mendel's laws of dominant or recessive inheritance[14][15]. For the disease to manifest clinically, an individual may need a mutant *FECH* allele on one copy of chromosome 18 that reduces FECH activity by half, and one low-expression normal *FECH* allele such as IVS3-48C on the other copy of chromosome 18 that reduces FECH activity by approximately 20%[14][15]. Our study of a Chinese EPP pedigree suggests that the disease shows the same pattern of inheritance as reported in non-Chinese pedigrees.

We discovered other two *FECH* SNPs, c.921 A>G and IVS8-61_-62delCT, in our

pedigree members. To our knowledge, this is the first report of IVS8-61_-62delCT in China. The pedigree short tandem repeat SNP genotyping results suggest that c.757_761delAGAAG, c.921 A>G, and IVS8-61_-62delCT belong to the same haplotype. Nevertheless, in this pedigree, c.921 A>G and IVS8-61_-62delCT did not affect the clinical manifestations of EPP (Figure 5).

In this study, we have described an EPP-associated *FECH* haplotype, comprising a deletion mutation (c.757_761delAGAAG) and 3 SNPs (IVS3-48T>C, c.921A>G and IVS8-61_-62delCT). This is also the first report of c.757_761delAGAAG and IVS8-61_-62delCT in Chinese. Our analysis expands the spectrum of *FECH* gene mutations in Chinese, confirms the pathogenicity of c.757_761delAGAAG and verifies that the pattern of EPP inheritance in Chinese is the same as that in non-Chinese. In addition, we found that the IVS3-48C allele is markedly more frequent in Chinese than in Africans and Caucasians.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the 900th Hospital of the People's Liberation Army Joint Service Support Force, and informed consent was obtained from participant.

Consent for publication

The patient has signed the consent for publication.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author

upon reasonable request.

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Conflicts of interest

The authors declare no conflict of interests.

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Authors' contributions

Dongliang LI conceived and designed the experiments, and wrote the manuscript. Xiaoqing LIN performed the experiments. Xiulan AO, Jian FANG, Zhiqiang ZHANG analyzed the data. Zhiyong ZHENG, Yongqin YAN collected samples.

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Figure 1. Cutaneous manifestations in the proband. (a) Pigmentation and hyperkeratinization were visible on the patient's neck. (b) Current and healed skin lesions were present on both knees.

Figure 2. Imaging results of the proband.Upper abdominal computed tomography angiography showed hepatic cirrhosis, splenomegaly with portal hypertension, and esophageal varices.

Figure 3. Liver pathology in the proband. Hematoxylin and eosin staining was performed on liver biopsies, and micrographs showed (A) micronodular liver cirrhosis and mild chronic inflammation (×200),(B) intrahepatic bile duct thrombosis (×200),(C)intrahepatic bile thrombus and lipofuscin deposition in hepatocytes (×400),(D)reticular fiber staining indicative of ductal fibrosis (×100).(E) the tubular or granular liver tissue showing bright red birefringence under polarizing lenses,with the Maltese cross visible.

Figure 4. The pedigree of the proband.

Symptomatic; asymptomatic; proband; IVS3-48T>C, normal allele; IVS3-48T>C, mutant allele; IVS8-61_-62delAG, normal allele; IVS8-61_-62delAG, deletion mutant allele; c.757_761delAGAAG, normal allele; c.757_761delAGAAG, deletion mutant allele; c.921A>G,normal allele; c.921A>G, mutant allele

Figure 5. Mapping of 4 mutations in the *FECH* gene. Genotypes of c.757_761delAGAAG (rs780942159) in a (A) heterozygote (proband) and (B) homozygote (mother). Genotypes of (C) IVS3-48T>C (rs2272783) in a heterozygote(proband), (D) c.921A>G (rs536560) in a

heterozygote, and (E)IVS8-61_-62delCT (rs60626363) in a heterozygote(proband).

Figure 1

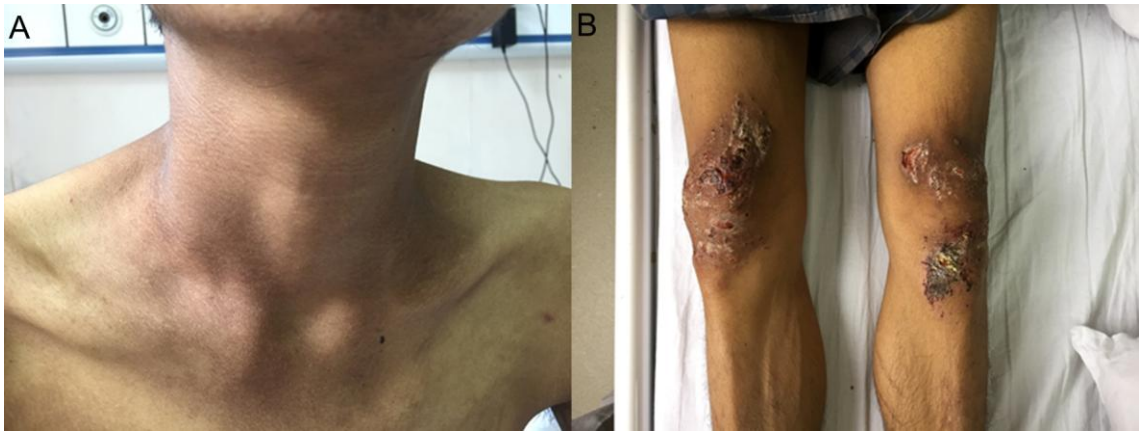


Figure 2

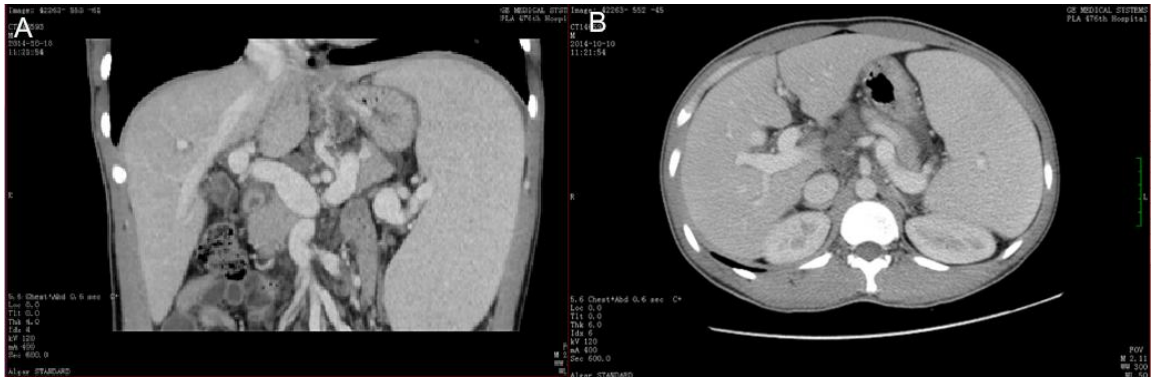


Figure 3

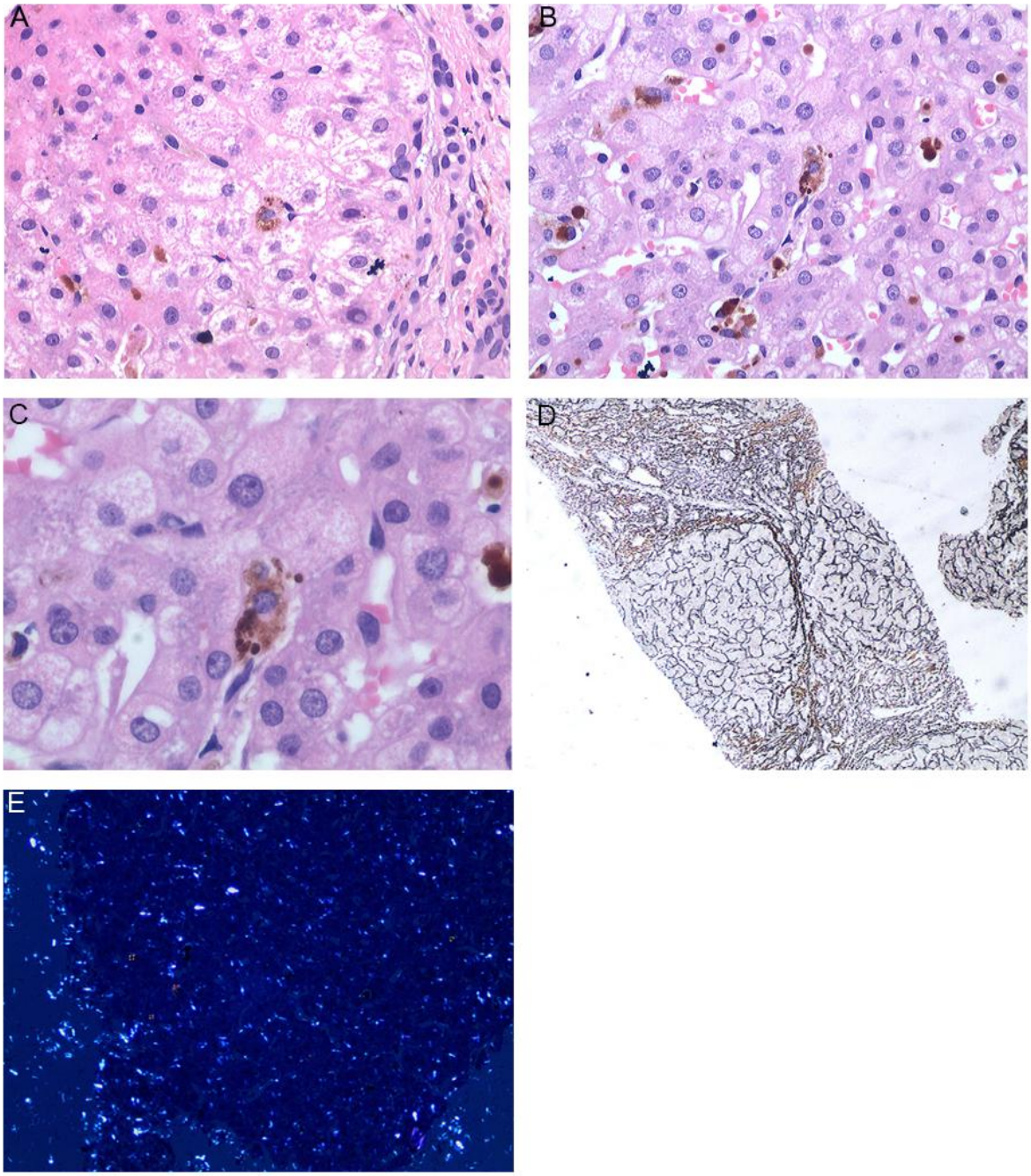
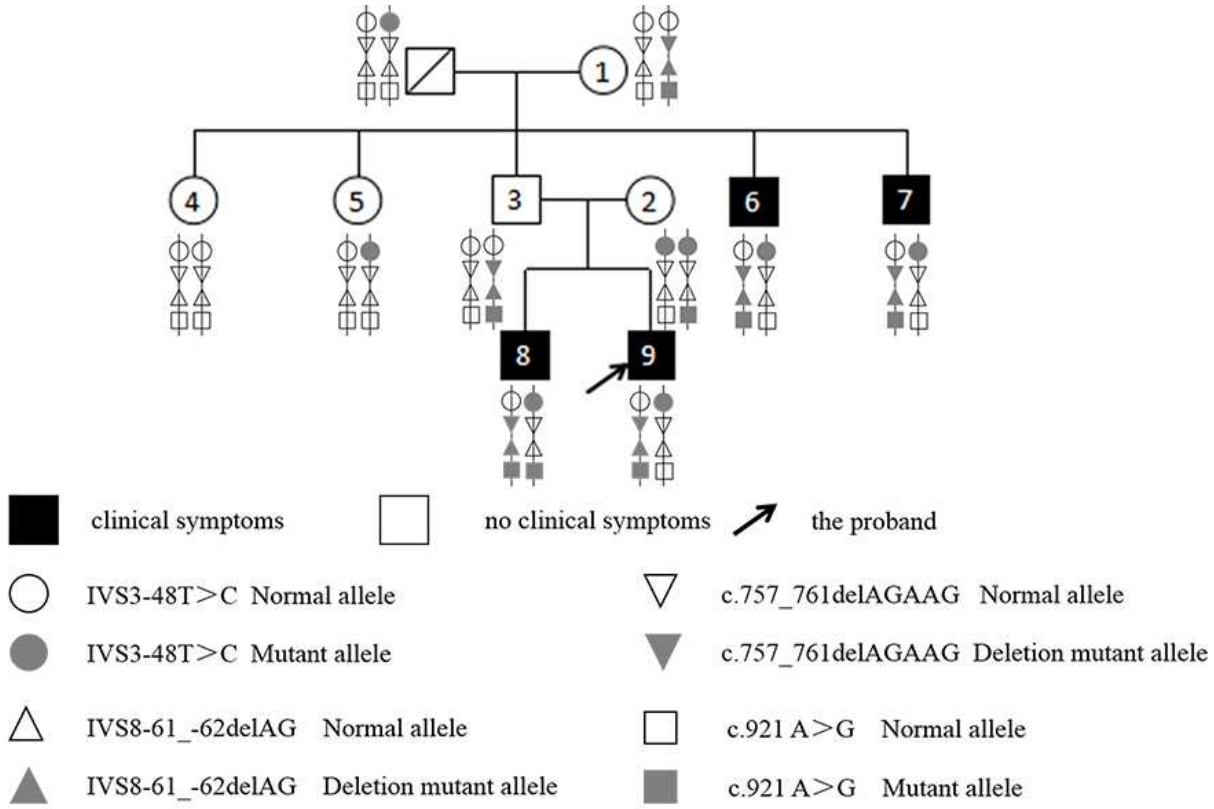


Figure 4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7
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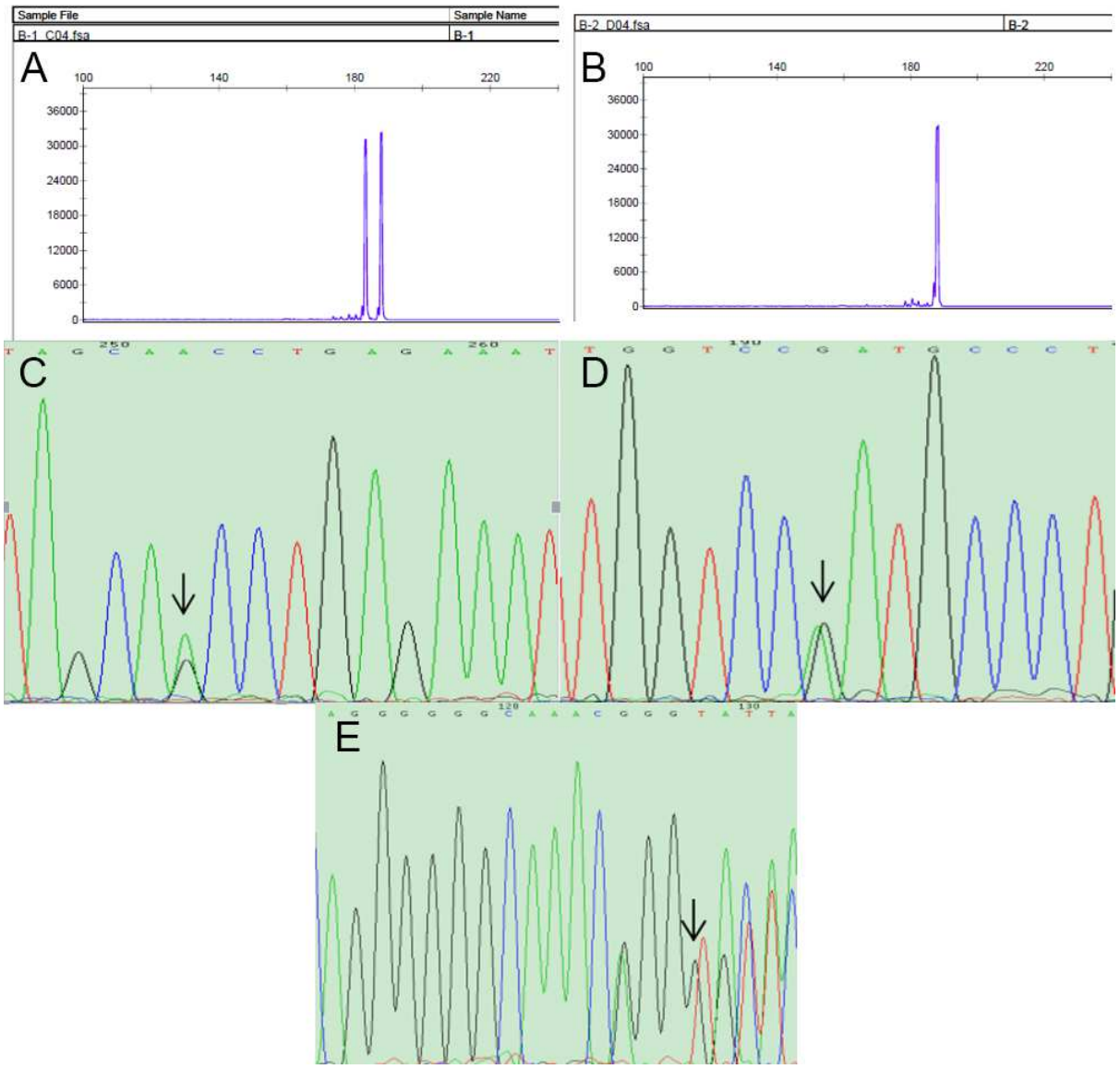
IVS3-48T>C Mutant allele
c.757_761delAGAAG Normal allele
c.757_761delAGAAG Deletion mutant allele
IVS8-61_-62delAG Normal allele
IVS8-61_-62delAG Deletion mutant allele
c.921 A>G Normal allele
c.921 A>G Mutant allele

Figure 5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B-2
B
C
D
E

Table 1. Methods to genotype loci related to erythropoietic protoporphyria in a Chinese pedigree

Locus	Primers	Amplicon length (bp)	Testing methods
rs780942159	F.AATGAAATCACCCAATCCTCTATC R.CTCTTTATCCTCCTTTCTTGTTACTCA	358	Capillary electrophoresis using reverse primer labeled with fluorescein
rs2272783	F.GAAGCATTTAGCATCTACTACTCTTTTG R.AAGGCTTAATCTTGTTAGGCTCTCTA	188	Sanger sequencing from forward primer
rs536560	F.TCTTCCTCCCCCTCTCACAAA R.TTTAGCTGCAATGACAGAAACTGT	286	Sanger sequencing from forward primer

[a] Amplicon length is based on human genome Hg19 deposited in the University of California Santa Cruz human genome database (http://genome.ucsc.edu/).

Abbreviations: F, forward primer; R, reverse primer;

Table 2. Genotyping of loci related to erythropoietic protoporphyria in a Chinese pedigree

No.	Relation to proband	Cutaneous manifestations	rs780942159 c.757_761delAGAAG	rs2272783 IVS3-48T>C	rs536560 c.921 A>G	rs60626363 IVS8-61_-62delCT
1	Grandmother	No	-/AGAAG	T/T	A/G	-/CT
2	Mother	No	AGAAG/AGAAG	C/C	A/G	CT/CT
3	Father	No	-/AGAAG	T/T	A/G	-/CT
4	Aunt1	No	AGAAG/AGAAG	T/T	A/A	CT/CT
5	Aunt2	No	AGAAG/AGAAG	T/C	A/A	CT/CT
6	Uncle1	Yes	-/AGAAG	T/C	A/G	-/CT
7	Uncle2	Yes	-/AGAAG	T/C	A/G	-/CT
8	Brother	Yes	-/AGAAG	T/C	G/G	-/CT
9	Proband	Yes	-/AGAAG	T/C	A/G	-/CT