Clinical, pathological, and genetic study of three cases of erythropoietic protoporphyria diagnosed in childhood

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Abstract

Objective: We investigated the clinical characteristics, liver pathology, ferrochelatase gene (FECH), and an intronic polymorphism at IVS3-48 in 3 cases from 2 families with erythropoietic protoporphyria (EPP) diagnosed in childhood.

Methods: Liver biopsies were performed in sibling cases, and pathological findings were evaluated along with clinical course. Genetic analyses were conducted in 3 cases and the results obtained were compared with clinical findings.

Results: Photosensitivity was observed in all cases and liver complications in the sibling cases. Pathological study of the sibling cases revealed mild liver fibrosis in the elder sister at 14 years of age, while severe fibrotic change with piecemeal necrosis and bridging fibrosis was found in the younger brother at 22 years of age. This difference in pathological severity between them was considered to be due to a difference in free erythrocyte protoporphyrin (FEP) levels. A heterozygous deletion of exon 4 (c.339delA) was found in the sibling cases. The other patient, who had photosensitivity without liver dysfunction, possessed a de novo heterozygous missense mutation in the last base of exon 4 (c.463G>C). All the patients had the IVS3-48T>C polymorphism, which is associated with splicing abnormality.

Conclusion: These findings suggest that FEP levels are associated with the progression of liver diseases and should therefore be monitored during regular follow-up in EPP patients. Sequence analysis of FECH and the intronic polymorphism at IVS3-48 should be considered on early diagnosis and therapeutic intervention to improve the prognosis.

Introduction

Erythropoietic protoporphyria (EPP, OMIM 177000) is an inherited metabolic disorder caused by a deficiency of ferrochelatase (FECH; EC4.99.1.1), which is the last enzyme in the heme biosynthetic pathway, catalyzing the conversion of protoporphyrin IX to heme. Erythropoietic protoporphyria has been reported worldwide, and the prevalence was estimated to be approximately 1:75,000 to 1:200,000. It has a complicated pattern of inheritance. In the majority of patients with EPP, ferrochelatase activity is impaired by the combination of a mutated FECH allele leading to a marked decrease in activity and a common hypomorphic allele (IVS3-48C), which is called “pseudo-dominant” inheritance. A few cases of EPP inherited in an autosomal recessive pattern have also been reported.

The characteristic symptoms of EPP are skin and liver manifestations caused by the accumulation of excess protoporphyrin IX in erythrocytes, skin, and liver. Photosensitivity is a common symptom, and other skin manifestations include blisters, hyperpigmentation, and hypopigmentation. Liver involvement is a significant complication, and patients may develop cirrhosis with histological findings of fibrosis and nodules.

Keywords: erythropoietic protoporphyria (EPP), ferrochelatase (FECH), photosensitivity, liver dysfunction, child

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sensitivity in sun-exposed skin is the most common symptom, which appears in early childhood. Hepatic involvement, including cholelithiasis and mild abnormalities of liver function, may occur in 10% to 20% of EPP cases. The terminal phase of EPP-associated hepatobiliary disease such as liver failure requiring liver transplant can develop in 2% of EPP cases. Therefore, regular follow-up of liver function and free erythrocyte protoporphyrin (FEP) levels are recommended. The association between EPP and liver involvement has been clarified. Autosomal recessive inheritance, null mutation, and a family history of EPP-related liver disease have been suggested to cause liver dysfunction. However, the precise pathology and predictors of liver involvement have not been revealed.

The present study describes the clinical characteristics, including genotype, in patients with EPP. The correlation among serum protoporphyrin levels, the progression of liver complications, and associated pathological findings was evaluated.

Materials and Methods

(Patients)

Three young patients (2 families) with EPP were recruited. Two were siblings (patients 1 and 2), and the other was a sporadic case (patient 3).

Patient 1, a Japanese girl, was 12 years old when she was referred to our hospital. She was born at 38 weeks of gestational age. She had an operation for congenital heart disease (ventricular septal defect, atrial septal defect, and pulmonary hypertension) when she was 8 months old. Elevated levels of transaminase were observed at the age of 10 months. Although she underwent detailed examination at several hospitals, tests, including those for viral hepatitis, were all negative. The elevated levels of liver transaminase were thought to have been caused by the blood transfusion she had received during heart surgery. She was referred to our hospital at the age of 12 years. We found that she had mild solar dermatitis after sun exposure for several hours. We analyzed her porphyrin levels in red blood cells, feces, and urine. The normal level of FEP is < 100 μg/dL, and in feces it is < 1,830 μg/day. She had a high protoporphyrin level in erythrocytes (3,562 μg/dL) and in feces (24,940 μg/day). The diagnosis was EPP based on the high levels of protoporphyrin in her erythrocytes and feces, and photosensitivity. She was started on ursodeoxycholic acid, but her elevated liver transaminase levels showed no improvement. A subsequent liver needle biopsy was performed at 12 years of age. After beginning the use of sunscreen and β-carotene at the age of 14 years in addition to ursodeoxycholic acid, her FEP levels decreased to less than 3,000 μg/dL and the levels of liver transaminase normalized (Fig. 1, upper panel).

Patient 2 was the younger brother of patient 1 and was 9 years old at the time of their initial visit. He was born at 39 weeks of gestational age. He also suffered mild photosensitivity after the age of 4 years. When the elder sister was diagnosed with EPP, his protoporphyrin levels were also analyzed. He also had a high level of FEP (2,779 μg/dL) without liver dysfunction. When he
complained of abdominal pain, and was admitted to our hospital at the age of 10 years, elevated levels of liver transaminase were observed for the first time. Chlorpromazine hydrochloride was administered for his severe abdominal pain, which was very effective. Avoidance of sun exposure had been recommended for him after the diagnosis. He was started on ursodeoxycholic acid at the age of 12 years. His FEP levels were lower than that of his sister at the beginning of follow-up (Fig. 1, lower panel). However, his FEP levels increased beyond those of his sister after the age of 15 years. Furthermore, his liver transaminase levels increased with increase in FEP levels. A liver biopsy was performed at the age of 22 years. The FEP level in the father (120 µg/dL) was slightly higher than the normal value without any symptoms. The FEP level of the mother was normal (<100 µg/dL).

Patient 3 was aged 13 years. He was born at 40 weeks of gestational age by cesarean section due to cephalopelvic disproportion. He complained of photosensitivity from the age of 5 years. His skin constantly showed redness and swelling, like a burn. Erythropoietic protoporphyria was diagnosed when he was 10 years old based on a high protoporphyrin level (erythrocytes : 2,310 µg/dL, feces : 2,121 µg/day). A very low level of porphobilinogen (0.5 mg/day) was detected in his urine. His liver transaminase levels were normal during the 4-year follow-up. He had no family history of liver dysfunction or photosensitivity.

The laboratory findings of the 3 patients are shown in Table 1. The results of the porphyrin analyses are shown in Table 2.

(Mutation analysis)
The human FECH gene is composed of 11 exons. It has been mapped to chromosome 18q21.3 and has already been cloned\(^{10,11}\). We performed a mutation analysis of the FECH gene in these patients and their parents. The intronic polymorphism IVS3-48 T>C was also analyzed in all patients and their available relatives. The study was approved by the ethical committee of Tokyo Medical University and informed consent was obtained from all the patients. Genomic DNA was isolated from peripheral blood leukocytes by using the QIAmap DNA kit (Qiagen, Japan). Using genomic DNA as a template, specific polymerase chain reaction (PCR) was performed to amplify the FECH gene. We designed primers for all the exons near the intron concerned based on GenBank sequences (GenBank accession no.

### Table 1  Laboratory findings in 3 patients with EPP

<table>
<thead>
<tr>
<th></th>
<th>Normal range</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
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<td>WBC (×10^3/μL)</td>
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<td>4,700</td>
<td>4,900</td>
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<td>RBC (×10^6/μL)</td>
<td>370-540</td>
<td>502</td>
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<td>465</td>
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<tr>
<td>Hb (g/dL)</td>
<td>11.0-17.0</td>
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<td>11.0</td>
<td>11.6</td>
</tr>
<tr>
<td>Plt (×10^3/μL)</td>
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<td>13.7</td>
<td>13.6</td>
<td>19.0</td>
</tr>
<tr>
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<td>11</td>
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<td>T-Bil (mg/dL)</td>
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<td>0.95</td>
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<td>I-Bil (mg/dL)</td>
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<td>TTT (U)</td>
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<td>2.1</td>
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<tr>
<td>ZTT (U)</td>
<td>2-12</td>
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<td>9.9</td>
<td>6.8</td>
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<td>Type 4 collagen (ng/mL)</td>
<td>&lt;150</td>
<td>180</td>
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<td>180</td>
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<td>Bile acid (µmol/L)</td>
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<td>10.9</td>
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<td>–</td>
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<td>Fe (µg/dL)</td>
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<td>TIBC (µg/dL)</td>
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<tr>
<td>Ferritin (ng/mL)</td>
<td>6-138</td>
<td>11.8</td>
<td>8.1</td>
<td>29.7</td>
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</table>

WBC ; white blood cell, RBC ; red blood cell, Hb ; hemoglobin, Plt ; platelet, AST ; asparate aminotransferase, ALT ; alanine aminotransferase LDH ; lactate dehydrogenase, γ-GTP ; gamma-glutamyl transpeptidase ALP ; alkaline phosphatase, T-Bil ; total bilirubin, D-bil ; direct bilirubin, I-bil ; indirect bilirubin, TTT ; thymol turbidity test, ZTT ; zinc sulfate turbidity test, Pb ; plumbum (lead)
The primers used are shown in Table 3. Each PCR contained 50 pmol forward and reverse primers, 12.5 μmol each dNTP mixture (Takara Bio Inc, Tokyo, Japan), 0.5 μl dimethyl sulfoxide, and 1.25 U Ex Taq DNA polymerase (Takara Bio Inc, Tokyo, Japan) in a total volume of 50 μl with distilled, deionized water. The amplification profile was 3 min at 95 degrees Celsius for initial denaturation, followed by 30 cycles of denaturation at 94 degrees Celsius for 30 sec, an annealing temperature of 55–72 degrees Celsius for 30 sec and 72 degrees Celsius for 60 sec, with a final extension at 72 degrees Celsius for 7 min. Because exon 11 was so long, it was divided into 3 parts and analyzed by using 6 primers. After the PCR was performed, we purified the PCR products by using the Wizard® SV Gel and PCR clean-up system (Promega, USA). Sequence analysis was carried out by bidirectional sequencing (LIC 4200, LI-COR) with tailed dye primer IRD 800 M 13, forward (5'-CACGACGTGGATAAAGCAGC-3') and reverse (5'-CGATAAAATTTCCACACAGG-3').

### Results

(Pathological findings)

Percutaneous liver biopsies were performed for patient 1 at 12 years of age and for patient 2 at 22 years. In patient 1, the liver was found to be in the early stage of liver fibrosis. Hematoxylin-eosin (HE) staining demonstrated numerous deposits of brown pigment in hepatocytes and Kupffer cells (Fig. 2A). These brown deposits were negative for Berlin blue staining (Fig. 2B). Azan staining revealed fibrous expansion of portal fields and disruption of the hepatic lobules, with septum formation and pericellular fibrosis (Fig. 2C). Electron microscopic analysis demonstrated that there were needle-shaped deposits in the hepatocytes (Fig. 3A). Greatly enlarged mitochondria were also observed, which con-
tained pectinate material that was different from cristae (Fig. 3B). In patient 2, the liver showed chronic hepatitis with piecemeal necrosis, cholestasis, and progressive hepatic fibrosis. Infiltration of inflammatory cells was observed in the portal areas. Brown pigments were seen within hepatic lobules and small bile ducts. Deposition of brown pigments was also found in the Kupffer cells (Fig. 4A). These brown pigments were unstained by Berlin blue staining (Fig. 4B). Intense fibrosis with expansion of portal areas and marked bridging fibrosis with nodule formation were present (Fig. 4C).

(Mutation analysis)

Patient 1, 2, and their father harbored a heterozygous mutation, c.339delA (p. Lys113fs), in exon 4 of the FECH gene (Table 4). This frameshift mutation results in the creation of a stop codon at the position of amino acid no.144. Their mother had no mutation in the same part of exon 4. The intronic polymorphism IVS3–48C was identified in Patient 1, 2, and their mother.

In patient 3, a de novo heterozygous mutation, c.463G>C (p.Ala155Pro), in the last base of exon 4, and the intronic polymorphism IVS3–48C were found (Table 5).

Fig. 2  Liver pathology in patient 1
A : Large amount of brown pigment was present in hepatocytes and Kupffer cells marked by arrow. (H&E, 200×)
B : These brown deposits were negative for Berlin blue staining. (Berlin blue, 200×)
C : Septum formation and pericellular fibrosis were observed around portal area. (Azan, 200×)

Fig. 3  Electron microscopic analysis in patient 1
A : Electron microscopic analysis in patient 1 revealed many variably sized slender crystals in cytoplasm.
B : Enlarged mitochondria were identified (marked by arrow).
Neither his parents nor younger brother had the mutation in FECH. We could not analyze the intronic polymorphism or the erythrocyte protoporphyrin levels of his family members.

### Discussion

This study investigated the clinical features of EPP with the focus on fluctuations in protoporphyrin levels, liver complications, and FECH mutations, including the splice site modulator IVS3-48C.

We observed liver complications in the sibling cases sharing the frameshift mutation, whereas the sporadic case with the missense mutation didn’t develop liver disease. Interestingly, the sibling cases showed differences in severity of liver disease and FEP levels. A previous study reported that all the EPP patients who developed severe liver involvement had a null allele mutation in FECH, but patients with a missense mutation didn’t
develop liver complications\textsuperscript{12}. Other studies support the relationship between major structural alterations in the FECH protein resulting from nonsense, frameshift, or splice site mutations, and liver disease\textsuperscript{13,14}. Autosomal recessive erythropoietic protoporphyria may have a stronger tendency to cause severe liver complications than other forms of EPP\textsuperscript{15,16}. However, it has been reported that sibling cases with overt EPP showed differences in liver disease, in spite of the identical genotype\textsuperscript{17}. Moreover, several studies have reported an association between levels of FEP and liver damage. The FEP levels in EPP patients with liver involvement were markedly higher than those in patients without (2,134 ± 450 vs. 730 ± 112 µg/dL; $P < 0.001$)\textsuperscript{18,19}. The development and deterioration of liver disease in sibling cases could be explained by genotype and elevation of FEP levels.

In the human body, 80% of heme is synthesized in erythropoietic cells, 15% in liver parenchymal cells, and the rest is distributed in other tissues\textsuperscript{20}. Heme biosynthesis is impaired by the defect of FECH in EPP patients, so excess protoporphyrin accumulates in erythrocytes, liver, skin, and other tissues. Liver dysfunction is caused by the accumulation of protoporphyrin in hepatocytes and canaliculi, when the levels of protoporphyrin are too high to be excreted by liver function\textsuperscript{21}. An excess of protoporphyrin in the liver may form solid deposits, thus obstructing the flow of bile. Crystalline deposits of protoporphyrin also directly cause cell damage\textsuperscript{22}. Upon irradiation of erythrocytes containing large amounts of protoporphyrin, the cells release massive amounts of protoporphyrin into the blood, leading to acute onset, severe hepatobiliary disease\textsuperscript{23}. Erythropoietic protoporphyria-related liver disease varies in severity from mild to fatal. Mild forms of EPP-related liver disease are characterized by elevated levels of liver enzymes and cholestasis. Progressive forms of liver disease include liver fibrosis, cirrhosis, and liver failure requiring liver transplantation\textsuperscript{9}. The progression of EPP-related liver disease leads to worse outcomes.

The early stage liver fibrosis observed in patient 1 suggests that there can be significant hepatocellular damage, even when the clinical manifestations are not so severe during childhood. We observed many small deposits of brown pigments in the hepatocytes and Kupffer cells, resembling bile pigments or hemosiderin in both cases. These deposits were brown, even before staining, and negative for Berlin blue staining, which stains hemosiderin. The sibling cases didn’t have cholestasis. Therefore, the brown precipitates were thought to be crystals of protoporphyrin, as reported previously\textsuperscript{19,24,25}. The needle-shaped deposits in patient 1 were similar to those previously identified in patients with EPP as “needle like crystals” or “starburst patterns”\textsuperscript{26–28}. The general effects of EPP on mitochondria are enlargement and disappearance of cristae\textsuperscript{29}. In the histological analysis, we observed abundant pectinate material in the enlarged mitochondria. Rademakers et al described giant mitochondria with paracrystalline inclusions in EPP patients\textsuperscript{26}. Enlarged mitochondria may be associated with decreased activity of FECH in the heme synthesis pathway, which occurs in mitochondria.

At least 180 mutations in the \textit{FECH} gene have been registered in the Human Gene Mutation Database (HGMD; http://www.hgmd.org/). The mutation c.339delA possessed by the sibling cases here has already been reported, although that study did not provide any information about the liver damage involved\textsuperscript{30}. Wang described a patient who had the same mutation, c.463G>C, as patient 3, and noted that the mutation resulted in exon 4 skipping or acted as a missense mutation leading to decreased FECH activity, but the intronic polymorphism IVS3-48C was not mentioned\textsuperscript{31}. In the present study, all the patients carried a deleterious mutation on one allele of \textit{FECH} and IVS3–48C on the other allele. The IVS3–48C polymorphism in the \textit{FECH} gene has been reported to modulate the splice site and to pro-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Symptoms</th>
<th>FEP (µg/dL)</th>
<th>Mutation in \textit{FECH}</th>
<th>IVS3-48</th>
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</thead>
<tbody>
<tr>
<td>Mother</td>
<td>–</td>
<td>not tested</td>
<td>N.D.</td>
<td>not tested</td>
</tr>
<tr>
<td>Father</td>
<td>–</td>
<td>not tested</td>
<td>N.D.</td>
<td>not tested</td>
</tr>
<tr>
<td>brother</td>
<td>–</td>
<td>not tested</td>
<td>N.D.</td>
<td>not tested</td>
</tr>
<tr>
<td>Patient 3</td>
<td>+</td>
<td>2,310</td>
<td>c.463G&gt;C</td>
<td>T/C</td>
</tr>
</tbody>
</table>

FEP: free erythrocyte protoporphyrin, N.D.: not detected.

\begin{align*}
\text{c.463G>C; p.Ala155Pro} & \quad \text{IVS3-48 T/C}
\end{align*}
duce aberrantly spliced mRNA degraded by nonsense-mediated decay\cite{12,33}. The frequency of the polymorphism varies markedly among populations, in the range from less than 1% in West Africa to 45% in Japan\cite{1,2}.

The prevalence rate of EPP in Japanese has been considered to be higher than in other populations.

Patient 1 (elder sister) showed elevated levels of liver transaminase from the age of 10 months, whereas patient 2 (younger brother) did not exhibit liver damage, including elevated transaminase levels, in early childhood. When patient 1 was 8 months old, she underwent an operation for congenital heart defects. Several reports showed that EPP patients may develop phototoxic injury during surgical procedures. Therefore, the use of protective light filters is recommended\cite{4,11,335}. Because patient 1 had not been diagnosed with EPP at the time of her operation, surgical lights without protective filters may have caused phototoxic injury to skin and erythrocytes, leading to liver damage. After her mid-teens, the liver transaminase levels in patient 1 (elder sister) normalized as her FEP level decreased (< 3,000 µg/dL), whereas those in patient 2 (younger brother) and liver fibrosis seriously deteriorated as FEP levels increased (> 3,000 µg/dL), although avoidance of sun exposure and the use of sunscreen were equally advised to both of them. Genetic factors, FEP levels, and other factors such as difference in sex may also have contributed to progression of liver damage in the boy.

In conclusion, genetic analysis of the \textit{FECH} gene and the intronic polymorphism at IVS3-48 should be considered in early diagnosis and therapeutic intervention to improve the prognosis of EPP. Monitoring FEP levels in regular follow-up is effective in evaluating progression of liver damage.

Acknowledgments

This work was supported by the Private University Strategic Research Based Support Project (S1311016) from the Ministry of Education, Culture, Sports, Science, and Technology in Japan, and there are no conflicts of interest related to this study. Informed consent was obtained to perform this study from the patients reported in this manuscript, and they agreed to the publication of this study. The authors thank the Department of International Medical Communications of Tokyo Medical University for editing the manuscript.

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小児期に診断された骨髄性プロトポルフィリン症3症例における臨床的、病理学的、遺伝学的特徴の検討

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【要旨】小児期に診断された骨髄性プロトポルフィリン症（EPP）の2家系3症例において臨床的、病理学的および遺伝学的特徴の検討を行った。3例で光線過敏症を呈し、姉弟例で経過中に肝障害を認めた。肝生検は姉弟例で行い、姉は14歳時に肝生検を施行し軽度の肝線維化を認め、弟は22歳時に施行しpiecemeal necrosisと架橋性線維化を伴う著明な肝線維化を呈していた。姉は遊離プロトポルフィリン（FEP）の低下とともにトランスアミナーゼが正常化、弟はFEPの上昇とともに肝障害の著明な進行を認める。遺伝学的検査では、姉弟例でFECH遺伝子のexon4領域に既知のフレームシフト変異、孤発例ではexon4の終端に既知のミスセンス変異をheterozygousに認め、いずれの症例も対立アリルにlow expression alleleであるIVS3-48Cを同定した。以上の結果から、EPP症例のフォローにおいて、FEPのモニターを行い、上昇傾向を示した際には肝障害の発症、増悪に注意する必要があり、早期診断、治療介入による予後改善のためにFECH遺伝子解析およびIVS3-48T>Cの多型解析を考慮すべきであると考えられた。

（キーワード）骨髄性プロトポルフィリン症、FECH、IVS3-48、光線過敏症、肝障害