A new ferrochelatase mutation combined with low expression alleles in a Japanese patient with erythropoietic protoporphyria

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ABSTRACT

We investigated the molecular defect of the ferrochelatase gene in a Japanese patient with erythropoietic protoporphyria (EPP), and identified a novel 16 base pair (574–589) deletion within exon 5. This deletion resulted in a frame-shift mutation and created a premature stop codon at amino acid position 198. The same molecular defect was also identified in his mother and a brother who had symptomatic EPP, but not in his father who was asymptomatic. The subjects with EPP were homozygous for the low expression haplotype, while his father was heterozygous for this haplotype. These results indicate that the combination of a 16 base pair deletion and low expression of the wild-type allelic variant is responsible for EPP in this pedigree.

INTRODUCTION

Erythropoietic protoporphyria (EPP) is known to occur as an autosomal dominant disease, with a low clinical penetrance, due to a reduced activity of ferrochelatase (protoheme ferro-lyase; EC 4.99.1.1) [1], the terminal enzyme of the heme biosynthetic pathway, which catalyzes the insertion of a ferrous iron into protoporphyrin IX to form protoheme. Patients with EPP are clinically characterized by painful photosensitivity due to accumulation of protoporphyrin in the skin, and high levels of free protoporphyrin in erythrocytes, plasma and stool [2]. While most EPP patients are associated with skin photosensitivity only, some (5% to 10%) develop hepatic failure due to massive hepatic accumulation of protoporphyrin [3].

Since the sequence of ferrochelatase cDNA was published by Nakahashi et al. [4], more than 60 different kinds of molecular defects of ferrochelatase have been reported. In this study, we report the molecular cloning and nucleotide sequencing of ferrochelatase cDNA from a patient with EPP who was heterozygous for the gene defect, a 16-base deletion within exon 5. This defect was shown to be inherited from the proband’s mother, and resulted in the production of a truncated protein.

Previously, it was reported that the low expression of a wild-type allelic variant trans to a mutated ferrochelatase allele is generally required for clinical expression of EPP [5]. In this study, we also evaluated the set of three intragenic polymorphisms [-251G, IVS1-23T, IVS2satA9; G-T-A9] which were associated with the low-expressed allele. The proband and his siblings with EPP carried a deletion on an allele and three polymorphisms on both alleles.

METHODS

Patient and family members

The patient was a Japanese male, 27 years of age, with cutaneous photosensitivity characteristic of EPP, which started from early childhood. He was admitted to hospital complaining of upper abdominal pain and jaundice, additional to the cutaneous photosensitivity, at the age of...
21. Symptomatic EPP was also found in three generations in the family (Figure 1). Protoporphyrin in red blood cells (8779 µg/dl; normal 30–86 µg/dl) and in stool (6800 µg/dl; normal < 1830 µg/dl) were markedly elevated. Fluorescence examination of the proband’s blood, urine and stool was positive for porphyrins, as were blood samples from his mother and brother, who also showed cutaneous signs of EPP. There were several maternal relatives who had a history of hepatic disease. The father of the proband and paternal relatives had no signs of EPP, or liver disease. Informed consent for all

The first strand of cDNA was synthesized using SuperScript RNaseH reverse transcriptase (Gibco BRL, Gaithersburg, MD, U.S.A.), primed with random hexa-

Figure 1 Pedigree of the Japanese family with EPP
The proband is indicated by an arrow. □ O, normal; ■ ●, cutaneous photosensitivity; *, hepatic disorder; (age) in 1993.

Cell cultures and Northern blot analysis
Isolation of lymphocytes, transformation of cells with Epstein-Barr virus (EBV), and cultivation of lymphoblastoid cells were performed, as described previously [6]. Total RNA was isolated from lymphoblastoid cells, which were from the patient, the brother and an unrelated control subject, according to the method of Chirgwin et al. [7]. Total RNA (10 µg) was analysed by Northern blot analysis, as described previously [8]. Cloned normal human ferrochelatase cDNA was inserted into the PsI site of pGEM 3Z vector (Promega, Madison, WI, U.S.A.). A radioactive riboprobe of antisense ferrochelatase mRNA was synthesized according to the method of Melton et al. [9]. Hybridized Zeta probe filters were treated with RNase A (1 µg/ml) for 7 min at 37 °C and then washed under stringent conditions. Ferrochelatase mRNA was visualized by exposing the membrane to X-ray film, and its level was quantitated using a Bio Image analyser.

Synthesis of ferrochelatase cDNA
The first strand of cDNA was synthesized using SuperScript RNaseH reverse transcriptase (Gibco BRL, Gaithersburg, MD, U.S.A.), primed with random hexa-

ers. The entire coding region of ferrochelatase cDNA was amplified by PCR, using the following two synthetic oligonucleotides as primers. They are 5'-GAGGCTGCCCAGAGCA-3' and 5'-TTTGGCTAAACAGCGGGTG-3', corresponding to the 5'- and 3'-untranslated region of ferrochelatase cDNA respectively [4]. PCR was carried out using a DNA Thermal Cycler (PerkinElmer Cetus, Norwalk, CT, U.S.A.) with AmpliTaq DNA polymerase (PerkinElmer Cetus), employing a thermal cycle program, as described previously [8]. PCR was performed three times in separate experiments, and further steps were processed separately as well.

Cloning and sequencing of cDNA
The PCR products were purified using a Gene Clean kit (BIO101 Inc., LaJolla, CA, U.S.A.), followed by cloning into pGEM-T Easy Vectors (Promega, Madison, WI, U.S.A.). DNA sequencing was carried out by the dideoxy chain-termination method using a genetically engineered T7 DNA polymerase (SequiTherm Long-Read Cycle Sequencing Kits LC; Epicentre Technologies, Madison, WI, U.S.A.).

Nucleotide sequence analysis of amplified ferrochelatase genomic DNA
Genomic DNA was isolated from lymphoblastoid cells of the proband, the father, the mother and the brother, and digested with EcoRI. Regions containing molecular defects found in ferrochelatase cDNA were amplified by PCR. The PCR was carried out using specific oligomers, designed based on the reported sequence [10], which were primer FC977: 5'-tattatagaAGCGCAAACTGAA-3' (intron 2–exon 3) and FC978: 5'-ccccactacCTGTGGGGGGG-3' (intron 4–exon 4). The PCR primers used for the deletion are shown in Figure 3. The region containing these intragenic polymorphisms was amplified on genomic DNA from the proband, the father, the mother and the brother by PCR. The primers for +251G/A in the 5' promoter region are: 5'-AGGCA-ACTTGGAGAGACTG-3' and 5'-GGACAGACGCCAC-ACCTACGGG-3' [10]. 5'-GGATCCAGTGGGAGGGGG-3' and 5'-GAGGGCGAGAGGAGGAGG-3' for IVS-23'T/C [11] in intron 1, and 5'-ATTGTGGCACCACACTCAG-3' and 5'-GCACCTTACATTCACTGGCAGA-3' for the di-nucleotide repeat called IVS2insAn in intron 2 [12] respectively. Cloning followed by sequence analysis was carried out in the same way described above.

Expression of mutant ferrochelatase cDNA
The Vsp1–BamHI or BamHI–EcoRI fragment of wild-type mature ferrochelatase cDNA in prokaryotic expression vector pAR-HF [13] was replaced with the O1 or O4 mutant cDNA fragment respectively. They were transformed into Escherichia coli BL21 (ER3) for expression of ferrochelatase proteins, as described previously [13].
Western blot analysis and ferrochelatase assay
Ferrochelatase protein expressed in E. coli was analysed by Western blot analysis, as described previously [11]. Detection of the specific immune complex was carried out using the ECL® Western blotting detection system (Amersham Life Sciences, Bucks., U.K.). Ferrochelatase activity in the E. coli transformed with human ferrochelatase cDNA was determined by the method of Rossi et al. [15], as described previously [13].

RESULTS
Cloning and sequencing analysis of ferrochelatase cDNAs from the patient
Nucleotide sequence analysis of the cloned ferrochelatase cDNA of the proband revealed three point mutations (O1, O2 and O3) and a 16 base pair (574–589) deletion, termed the O4 deletion. The O1 mutation (A287→G), which resulted in the amino acid substitution Gln96→Arg, was detected in all the clones analysed. Both the O2 mutation (G798→C) and the O3 mutation (G921→A) were present on the same allele, termed allele 1, and were silent mutations (Figure 2). The other allele (allele 2) contained the O4 deletion (Figure 3), which introduced a frame-shift mutation and created a premature stop codon at amino acid position 198 (Figure 2). The frequency between allele 1 and allele 2 from randomly picked clones was approximately 9:1 (Table 1), suggesting that the O4 deletion results in a ferrochelatase mRNA with a very short half-life.

Pedigree studies
The O1 and O4 deletions were also confirmed in PCR-amplified genomic DNA from the proband. The O4 mutation was additionally found in genomic DNA from the proband’s mother and brother (Table 2). The same mutation was also identified in an unrelated Caucasian without EPP (results not shown). The frequency between the normal sequence and the O4 deletion was approximately 1:1, in randomly picked clones from the proband, his mother and brother (Table 2). The O4 deletion was absent in the father (Table 2). These findings indicated that the O4 deletion has been transmitted to the proband from his mother.

Northern blot analysis
Since the transcript from allele 2 was suspected to be significantly lower than that from allele 1, we carried out Northern blot analysis to measure ferrochelatase mRNA levels in the proband and his brother, in comparison with a normal control. Two species of ferrochelatase mRNAs of 2.5 kb and 1.6 kb, consistent with the two polyadenylation sites in this mRNA [4], were detected in total RNA isolated from EBV-transformed lymphoblasts from the proband and his brother, as well as a normal control. The levels of the major 1.6 kb ferrochelatase mRNAs, quantified after standardizing the rRNA level, in the proband and his brother were approx. 10% and 25% compared with the normal control respectively (Figure 4).

Identification of three intragenic polymorphisms
Since the ferrochelatase mRNA level in the proband and his brother was significantly lower than half of the normal, the expression of both alleles was suspected to be decreased. We therefore carried out an investigation of the polymorphisms, which were reported to be strongly associated with the low-expressed allelic variant (Figure 5) [5]. The set of three intragenic polymorphisms [G-T-A9] was identified as a homozygous trait not only in the proband but also in his mother and brother. The father
Figure 3 O4 deletion in genomic DNA

(A) Partial sequence of ferrochelatase from proband's genomic DNA is shown. **Bold letters in the square show the position of the 16 base pairs (574–589).** (B) DNA sequence of ferrochelatase exons 5 and 6 (upper-case letters) and intron 5 (lower-case letters), surrounding the O4 deletion. Primer FC971 (570–559 in exon 5) and primer FC972 (629–674 in exon 6) were used for the first PCR using genomic DNA from the normal subject as a template. On the basis of the nucleotide sequence obtained from the first PCR, primer FC979 (513–542 in intron 5) was designed as the primer for PCR on genomic DNA.

Table 1 Proportion of two allelic products detected in cDNA from the proband

<table>
<thead>
<tr>
<th>Allele</th>
<th>Library 1</th>
<th>Library 2</th>
<th>Library 3</th>
<th>Total</th>
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<tr>
<td>1</td>
<td>5/6</td>
<td>12/12</td>
<td>14/17</td>
<td>31/35 (89%)</td>
</tr>
<tr>
<td>2</td>
<td>1/6</td>
<td>0/12</td>
<td>3/17</td>
<td>4/35 (11%)</td>
</tr>
</tbody>
</table>

was heterozygous for the set of polymorphisms, indicating that the proband and his brother inherited a wild-type allele with low expression from their father, and a mutant allele from their mother.

Table 2 Detection of the O4 deletion in genomic DNA from family members

<table>
<thead>
<tr>
<th></th>
<th>Proportion of clones with the O4 deletion</th>
</tr>
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<tbody>
<tr>
<td>Proband</td>
<td>4/8</td>
</tr>
<tr>
<td>Father</td>
<td>0/6</td>
</tr>
<tr>
<td>Mother</td>
<td>2/6</td>
</tr>
<tr>
<td>Brother</td>
<td>2/5</td>
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</tbody>
</table>

Figure 4 Ferrochelatase mRNA from lymphoblastoid cells

Ferrochelatase mRNA was examined by Northern blot analysis, as described in the Materials and methods section. (A) Ferrochelatase mRNA. (B) Ethidium bromide-stained agarose gel showing rRNA profiles. Lane 1, proband; lane 2, brother; lane 3, normal control.

Figure 5 The co-inheritance of a low-expressed ferrochelatase haplotype and a mutant allele

Haplotypes that are associated with the low-expressed alleles are in the dotted box, and those associated with the O4 mutant are in the black box. EPP patients are indicated with arrows.

Ferrochelatase activity expressed by the mutant cDNAs

To determine the effect of the O1 and O4 defects on enzyme activity, mutant ferrochelatase cDNA was expressed by transfection into E. coli cells. The expressed ferrochelatase activities were compared with the activities obtained by transfection of the normal ferrochelatase cDNA. Human ferrochelatase protein expressed in E.
Figure 6 Expression of human ferrochelatase in E. coli

Transfection and expression of normal, O1 and O4 mutant ferrochelatase cDNAs in E. coli were carried out as described in the Material and methods section. Upper panel: the amount of ferrochelatase expressed was determined by Western blot analysis. Lower panel: enzyme activity was expressed as the activity/mass ratio, standardized by the normal control. ‘Mock’ indicates E. coli transfected with plasmid only.

coli cells was specifically detected and quantified by Western blot analysis using a polyclonal antibody, which reacts with the human ferrochelatase but not with E. coli ferrochelatase. Human-specific ferrochelatase activity was calculated as an activity versus mass ratio (A/M). The O1 mutant showed 91.8% of the A/M ratio compared with that expressed by the normal ferrochelatase cDNA (Figure 6). Mutant ferrochelatase carrying the O4 deletion expressed no significant immunological protein, with null activity.

DISCUSSION

In this study, we demonstrated a novel molecular defect of the ferrochelatase gene in a Japanese male with EPP. A total of four mutations in the ferrochelatase gene have been identified in this subject. While the O2 and O3 mutations were silent, thus representing sequence polymorphisms, the O1 mutation and O4 deletion accompanied an amino acid substitution and frame-shift mutation respectively.

The O1 mutation, which resulted in an amino acid substitution, has been identified not only in the symptomatic proband, his mother and brother, but also in the asymptomatic father. It was also found in an unrelated healthy Caucasian without any symptoms. Thus this mutation is unlikely to be related to EPP in this family. In fact, mutant cDNA containing the O1 mutation expressed in E. coli showed a significant amount of ferrochelatase protein with normal activity (Figure 6).

In contrast, the O4 deletion, which is a 16 base pair deletion involving nucleotide base pairs 574–589, and results in translation into a truncated protein, is exclusively found in subjects with EPP, but not in the asymptomatic father in this family. Thus it is highly likely that the O4 deletion is responsible for EPP in this family. Our expression studies in E. coli cells clearly showed that the mutant ferrochelatase with the O4 deletion was translated as no immunologically detectable protein or enzyme activity (Figure 6). The O4 deletion, which results in six abnormal amino acid residues after amino acid 192, caused a frame-shift mutation and formed a premature stop codon at amino acid position 198. As a result, allele 2 is translated as an abnormally short protein lacking a large portion of its C-terminal region (Figure 2). This C-terminal region is known to contain various residues, or sites essential for the function of ferrochelatase. It has been reported that His$^{295}$ in ferrochelatase is essential for metal binding, and that Asp$^{246}$ and Tyr$^{248}$ are also involved in metal binding in a synergistic manner [14]. His$^{295}$ was reported to contribute to the maintenance of the structure of the protein molecule [16]. Besides, it is known that human ferrochelatase contains critical cysteine residues at positions 403, 406 and 411, which comprise a [2Fe–2S] cluster, essential for enzyme activity [17,18]. This information suggests that the truncated protein, due to the O4 deletion in allele 2, does not have significant ferrochelatase activity because of the lack of a number of amino acids which are essential for enzyme activity.

It was reported that ferrochelatase mRNA levels decreased by haem depletion [19], thus impaired haem biosynthesis in the EPP patient might cause the failure to maintain ferrochelatase mRNA levels. Besides, the existence of a premature stop codon in the O4-derived mRNA is likely to reduce its stability. Ferrochelatase mRNA in the patient transcribed from allele 2 is unstable, because (i) the frequency of allele 2-derived transcript from the proband cDNA is approximately 10% compared with allele 1 (Table 1), and (ii) Northern blot analysis of total RNA prepared from lymphoblastoid cells showed a significant decrease in ferrochelatase mRNA in the proband and his brother (Figure 4). Although the proband and his brother were suspected to be heterogeneous in the molecular status, ferrochelatase mRNA levels, which were expected to be decreased to half, were about 10% and 25% compared with that of cells from a normal control (Figure 4). The result suggests that the low expression is not only due to the allele 2 having the O4 deletion, but also due to the normal allele 1. Recently, it has been reported that clinical expression required
coinheritance of a normal ferrochelatase allele with low expression together with a mutant ferrochelatase allele [5]. The proband, his mother and brother with EPP symptoms had the three polymorphisms [G-T-A9] of ferrochelatase, which were shown to bring low expression of the ferrochelatase allele (Figure 5). The father was heterozygous for the three polymorphisms, indicating that it was inherited from the father to the sons, because they carried the mutant allele from their mother.

It was characteristic for the patient and his symptomatic brother to develop this severe hepatic disorder. The molecular defects leading to a null allele were reported to be a common feature among EPP pedigrees with liver complications [20]. Since the novel mutant ferrochelatase found in this family expressed null enzyme activity in E. coli (Figure 6), it might be reasonable to suppose that the massive accumulation of protoporphyrin caused by markedly decreased ferrochelatase activity induced progressive liver failure.

Although the mechanism of low expression remains unclear, the frequency of the low expression ferrochelatase allele in the asymptomatic Caucasian population is estimated to be from 6.5 to 11.5% [5]. The extensive analysis of the relationship between clinical consequences and ferrochelatase deficiency by Schneider-Yin et al. [21] clearly showed that the combination of a mutated allele and a low expressed normal allele is responsible for the clinical complications observed in the patient with EPP. Moreover about 1% of the population was supposed to be homozygous. This is the first report of the three polymorphisms being identified in a homozygous trait, together with a unique frame-shift mutation caused by a 16 base pair deletion in exon 5 of the ferrochelatase gene from Japanese patients with EPP.

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