Porphyrias are a group of metabolic diseases which are mostly genetically determined and clinically heterogeneous. They are caused by a decreased activity of particular enzymes participating in the heme biosynthesis pathway, except for the synthase of δ-aminolevulinic acid. Porphyrias are classified as “hepatic” or “erythroid” on the basis of the tissue that is the predominant site of accumulation of metabolic intermediates. The eight major porphyrias have been classified into the 3 groups: 1) four acute hepatic porphyrias, 2) the single hepatic cutaneous porphyria (porphyria
cutaneous porphyrias, PCT, and 3) three erythropoietic cutaneous porphyrias. PCT is the most prevalent of all porphyrias, both genetic and acquired and acute intermittent porphyria (AIP) is the most common of genetic porphyrias [1–3]. Its estimated incidence is 2 to 5 in 100,000 in the United States and northern European countries [4]. Acute intermittent porphyria (AIP) is inherited in an autosomal dominant fashion and of a low clinical penetrance. A 50% decrease in the activity of porphobilinogen deaminase (PBGD) in the erythrocytes, and/or the liver and other tissues, is a metabolic defect responsible for AIP. PBGD catalyses the third stage of heme biosynthesis pathway, i.e. condensation of 4 molecules of porphobilinogen to hydroxymethylbilane [5–7].

An abnormal heme biosynthesis in AIP is due to mutations in the PBGD gene. The 10 kb gene is located in the region 11q24.1-11q24.2 on chromosome 11. It is composed of 15 exons of 39 to 438 bp in size. The gene has two promoters initiating the synthesis of two different mRNAs (due to alternative splicing of exon 1 and 2), which initiates two enzyme forms showing tissue specificity: the erythroid form present only in erythrocytes, and the housekeeping form found in non-erythroid tissues. The promoter active in the non-erythroid tissues is located in the borderline sequence of gene 5′ and is responsible for the formation of mRNA which contains 1250 bp exons 1 and 3–15 encoding for a common (non-erythroid) enzyme form composed of 361 amino acids (42 kDa). The other promoter, active only in erythrocytes, is located in intron 1, and is responsible for the formation of 1038 bp mRNA. It contains exons 2–15 encoding for the erythroid PBGD composed of 344 amino acids of 40 kDa in molecular weight. This isoform does not contain the first 17 amino acids at the end of NH2, the function of which is unknown [8–11].

Clinical and biochemical AIP manifestations are due to an altered activity of two isoenzymes encoded by a mutated gene. In 93–98% of cases, AIP families demonstrate a classical form in which both enzymes are defective. In patients with the so-called non-erythroid AIP, the PBGD activity is normal in erythrocytes but diminished in other tissues [7–9]. In approximately 80% of subjects affected with AIP, excretion of heme precursors is normal, and they do not develop any clinical manifestations [12, 13]. Symptoms usually occur in patients aged 18–40 years, mostly in women. The overt AIP, manifested as an attack or exacerbation, is induced by porphyrinogenic factors, i.e. certain drugs (e.g. barbiturates and sulfonamide antibiotics), and chemical compounds (paints, veneers, organic solvents), alcohol ingestion, tobacco consumption, starvation, stress, concomitant infections, and surgery. A delayed diagnosis of the AIP attack may lead to disability or even death [12, 14].

During attacks patients excrete considerable amounts of porphobilinogen (PBG) and δ-amino levulinic acid (ALA) and porphyrins in urine, but faecal porphyrins excretion is normal. In erythroid AIP, the erythrocyte PBGD activity is reduced during remission, but rises to a normal level during an attack. In asymptomatic patients, the enzyme activity is decreased. In non-erythroid AIP, the PBGD activity is normal in erythrocytes, but diminished in other tissues [7–9].

The aim of the present study was to characterise molecular errors in the PBGD gene in a group of Polish patients with non-erythroid AIP, and to evaluate the efficacy of the DNA sequencing method in the early diagnosis of this disorder.

**Material and Methods**

Twenty five patients, 17 women, 8 men (aged from 14 to 63), registered at the Porphyria Unit, Institute of Haematology and Transfusion Medicine, were members of 9 unrelated families from different parts of Poland. Urinary PBG and ALA excretion as well as PBGD activity in erythrocytes during remission were determined in all subjects.

Urinary PBG and ALA excretion levels were determined using Mauzerall and Granicka’s method [15]; erythrocyte PBGD activity was assessed using the method by Magnussen et al. [16].

DNA was isolated from the peripheral blood collected on EDTA. PCR reaction for exons 1 and 3–15 was performed using primers without GC-clamp, as described by Puy et al. [17]. In the PCR reaction, the following were used per 50 µL of a re-active mixture: 0.1 µg of genomic DNA, 5 pM of each primer, 200 µm dNTPs, a tenfold PCR buffer concentration (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 0.1% triton Mm-100; 50% glycerol v/v; pH 8.0), 2 mM MgCl2, 1 U Taq Polymerase (Biotools). Amplification programme was performed in 35 cycles of: 30 s denaturation at 95°C; 30 s annealing at 59°C, and 30 s elongation at 72°C.

Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit v.1.1 on the Hitachi 3730 Analyzer (Applied Biosystem, USA). Each tested fragment was sequenced towards sense and antisense. The sequence obtained was compared with the cDNA reference sequence from Gen Bank No M95623, numbered from the ATG sequence of the translation-initiation housekeeping codon of enzyme isoform, where A is nucleotide +1.
Results

In 9 assessed families (25 subjects) with non-erythroid porphyria, acute porphyria was diagnosed in 11 patients based on clinical manifestations and increased urinary excretion of PBG and ALA. No clinical features of the disease were found in 14 family members, including 10 subjects with a normal urinary excretion of heme precursors, 3 individuals with slightly increased values and 1 person without determination of urinary PBG and ALA excretion.

In 24 out of 25 patients from the investigated families, erythrocyte PBGD activity in the remission period was normal. In one of 25 subjects, the erythrocyte PBGD activity was decreased for no obvious reason (Table 1).

Table 1. Biochemical and molecular characterization of patients with non-erythroid AIP

<table>
<thead>
<tr>
<th>No family</th>
<th>Patient/sex/age</th>
<th>PBGD activity in RBC (nmol URO/mL RBC/h) 29.3–39.2</th>
<th>Urinary PBG (µmol/24 h) 0.95–15.0</th>
<th>Urinary ALA (µmol/24 h) 1.1–57.2</th>
<th>Clinical state</th>
<th>Mutation PBGD gene</th>
<th>Mutation consequence</th>
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<tr>
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<td>32.5</td>
<td>1536.2</td>
<td>1326.0</td>
<td>acute</td>
<td>33+1G &gt; A</td>
<td>67 bp retention, stop+41</td>
</tr>
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<td>2/M/63</td>
<td>32.5</td>
<td>843.8</td>
<td>768.9</td>
<td>acute</td>
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<td>67 bp retention, stop+41</td>
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<td></td>
<td>3/F/29</td>
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<td>6.7</td>
<td>21.1</td>
<td>asymptomatic</td>
<td>no mutation</td>
<td></td>
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<td></td>
<td>4/F/19</td>
<td>31.4</td>
<td>17.1</td>
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<td>33 + 1G &gt; A</td>
<td>67 bp retention, stop + 41</td>
</tr>
<tr>
<td></td>
<td>5/M/18</td>
<td>30.7</td>
<td>8.5</td>
<td>74.6</td>
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<td>no mutation</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1/F/41</td>
<td>30.9</td>
<td>233.9</td>
<td>186.3</td>
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<td>33 + 1G &gt; A</td>
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<td>322.2</td>
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<td>33 + 1G &gt; A</td>
<td>67 bp retention, stop + 41</td>
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<td>12.3</td>
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<td>33 + 1G &gt; A</td>
<td>67 bp retention, stop + 41</td>
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<tr>
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<td>6.2</td>
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<td>33 + 1G &gt; A</td>
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</tr>
<tr>
<td></td>
<td>6/M/12</td>
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<td>7.2</td>
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<tr>
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<td>1613.0</td>
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<td>ND</td>
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<td>180.5</td>
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<tr>
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<td>36.5</td>
<td>819.9</td>
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<td>acute</td>
<td>33 + 2T &gt; C</td>
<td>67 bp retention, stop + 41</td>
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<tr>
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<td>577.1</td>
<td>388.1</td>
<td>acute</td>
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<td>67 bp retention, stop + 41</td>
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<td>33 + (4–12) del AGTGCTGAG</td>
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<tr>
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<td>32.8</td>
<td>7.3</td>
<td>40.5</td>
<td>asymptomatic</td>
<td>no mutation</td>
<td></td>
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</tbody>
</table>

a nucleotides were numbered according to human PBGD cDNA (GenBank accession no M95623) with the A of the ATG initiation codon for the ubiquitous isoform as +1; PBGD – porphobilinogen deaminase, RBC – erythrocytes, URO – uroporphyrins, PBG – porphobilinogen, ALA-δ – aminolevulinic acid; bp – base pairs.
In 11 patients with acute porphyria who had raised both PBG and ALA urinary contents, and the investigation of the PBGD gene mutation confirmed the diagnosis of AIP in all cases (Table 1).

In 10 out of 14 asymptomatic subjects, both the PBG and ALA urinary excretion was normal and in 2 slightly increased, in 1 person there was only increase in ALA urinary excretion, and in the remaining one no measurement has been done.

In 6 out of 10 asymptomatic subjects the causative mutation responsible for AIP was found, and among these 6 subjects there were both who had an increase in the urinary excretion of PBG and ALA (Table 1).

In 9 families with non-erythroid AIP, all the recognized mutations were found in intron 1. They were 4 mutations, one novel unreported mutation: 33 + (4–12) del AGTGCTGAG in 60 years old female, and 3 known mutations, i.e. 33 + 1 G > A, 33 + 2 T > C, 33 + 5 G > C. Mutation 33 + 1 G > A was detected in 5 families; in 7 patients with acute porphyria, and in 6 subjects with a latent form. The mutation was excluded in 7 family members. In 6/7 subjects hem precursors excretion was normal. In 1/7 ALA excretion was slightly increased. Mutation 33 + 2 T > C was present in 2 patients with acute porphyria in 2 different families. In another family, one patient with acute porphyria was found to possess mutation 33 + 5 G > C. Each of these three mutations was responsible for the retention of 67 bp in intron 1 and the formation of stop codon in the position + 41.

In one family, the proband was found to have a novel unreported mutation, i.e. 33 + (4–12) del AGTGCTGAG. This deletion probably disturbs the process of exon 1 splicing. The mutation was excluded in one family member with normal urinary excretion of heme precursors.

All the mutations found in the present study represented those of the splice defect and resulted in abnormal exon 1 splicing.

**Discussion**

The pathophysiologic mechanisms of the clinical management of the porphyrinas are not yet wholly understood. AIP is caused by abnormal function of the PBGD, causing an accumulation of heme precursors, and then resulting in urinary overproduction of the porphyrin precursors, PBG and to a lesser extent ALA.

But, in clinically asymptomatic patients with non-erythroid AIP, the diagnosis of porphyria can be feasible only by using molecular methods since the excretion of PBG and ALA is normal in most patients, and the PBGD activity in erythrocytes is normal in all affected individuals.

The new finding of this study was the detection of four mutations in intron 1 of the PBGD gene in our AIP patients. There was one unreported novel mutation, 33+(4–12) del AGTGCTGAG, of an unknown biological mechanism present in a 60-year-old female. And we have revealed 3 previously described mutations, i.e. 33+1G>A, 33+2T>C, 33+5G>C, responsible for abnormal transcript splicing in the area of exon 1. Of 14 asymptomatic members of proband families 6 subjects were diagnosed with AIP, but in 8 the AIP was excluded based on the DNA sequencing method.

Reports have been published in the literature on over 300 different mutations of the PBGD gene responsible for AIP, of which only 11 mutations were found in families with non-erythroid AIP [18].

Until now, 73 PBGD reported gene mutations in the Polish population have been recognised in 167 families (375 subjects) of patients affected with the classical AIP [19]. Mutations in classical AIP were described in all the exons of the PBGD gene, except for exons 1 and 2. They were missense, nonsense, those at the splicing site, and frameshift mutations. Reports were published on 10 different mutations in exon and intron 1 leading to non-erythroid AIP. They included: 1 mutation in the promoter region evoking transcription weakness [20], 1 missense mutation in the translational initiating codon of a systemic isoform [21], 8 mutations yielding abnormal exon 1 splicing (leading to nucleotides retention and frameshift). The non-erythroid AIP was also caused by a frameshift mutation in exon 3, which resulted in the formation of the stop codon of the housekeeping isoform. Mutations in non-erythroid AIP were found in single families [18, 20–24]. In our previous study 10 new mutations in 16 families with AIP have been described, which were similar to those found in other populations [25]. Mutations in exon 1 and intron 1, responsible for non-erythroid AIP, did not reduce the PBGD activity in erythrocytes, since exon 1 copied from the systemic promoter does not encode for amino acids, and the transcript (mRNA) copied from an erythrocyte-specific promoter does not contain the sequence of exon 1 [26, 27]. Early detection of both non-erythroid and classical AIP is crucial in prevention; in the case of an attack, it allows initiating an adequate treatment. Implementation of DNA sequencing helps to early identify individuals affected with AIP in 95% of those assessed, in 91% of those with variegate porphyria, and in 85% of those with erythropoietic protoporphyria [28–30].
The register of porphyria patients, held by the Porphyria Unit, the Institute of Haematology and Transfusion Medicine, Warsaw, lists at present 340 families with the so-called classical AIP, and 9 families with non-erythroid AIP. The non-erythroid AIP is a very rare type of porphyria accounting for 1.5% of the total number of the families on the register. Diagnostic investigations performed in 18 patients with non-erythroid AIP revealed the presence of mutations occurring in the border-line sequence of exon 1 of the PBGD gene. They included mutations (33 + 1 G > A, 33 + 2 T > C, 33 + 5 G > C, 33 + (4–12) del AGTGCTGAC) disturbing the process of exon 1 splicing [20, 24].

In conclusion, the DNA sequencing based analysis is the only reliable method for detection of asymptomatic non-erythroid AIP patients with normal urinary excretion of heme precursors in affected families. The mutations found in Polish patients with non-erythroid AIP represented those of splice defect and resulted in abnormal exon 1 splicing.

References


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