MUTATION IN BRIEF

Molecular Study of the Hydroxymethlybilane Synthase Gene (HMBS) Among Polish Patients With Acute Intermittent Porphyria

Anita Gregor[1], Xiaoye Schneider-Yin[2], Urszula Szlendak[1], Albert Wettstein[2], Agnieszka Lipniacka[1], Urszula B. Rüfenacht[2], and Elisabeth I. Minder[2*]

[1]Porphyria center, Institute of Hematology and Blood Transfusion, Warsaw, Poland; [2]Zentrallabor, Stadtspital Triemli, Zürich, Switzerland

* Correspondence to: Elisabeth Minder, Zentrallabor, Stadtspital Triemli, Birmensdorferstrasse 497, CH-8063 Zürich, Switzerland; Tel.: +41-1-466-2320; Fax: +41-1-466-2744; E-mail: elisabeth.minder@triemli.stzh.ch

Contract Grant Sponsor: Roche Research Foundation, Contract Grant Number: 98-202.

Communicated by Mark H. Paalman

Acute intermittent porphyria (AIP), an autosomal dominant disorder of heme biosynthesis, is due to mutations in hydroxymethylbilane synthase (HMBS; or porphobilinogen deaminase, PBGD) gene. In this study, we analyzed 20 Polish patients affected by AIP and we were able to characterize seven novel mutations. A nonsense mutation (Y46X), two frameshift mutations (315delT and 552delT) and a 131bp deletion (nucleotides 992-1123) give rise to truncated proteins. A donor splice site mutation IVS12+2T>C predicts skipping of exon 12. A missense mutation (D61Y) was identified in two apparently unrelated patients with a clearly clinical indication of AIP. An inframe 3-bp deletion (278-280delTTG) results in the removal of V93 from the enzyme. In addition to the novel mutations, nine previously described HMBS gene mutations – R26H, G111R, IVS7+1G>A, R149X, R173Q, 730-731delCT, R225X, 982-983delCA and G335D – were identified in this cohort. Our results demonstrate that molecular analysis of the PBGD gene is a more reliable method comparing to enzymatic assay in the diagnosis of AIP. Although more than 170 different mutations are known to the HMBS gene so far, over 40% of all mutations identified among the Polish AIP patients of this study are novel mutations, indicating the heterogeneity of molecular defects causing AIP. © 2002 Wiley-Liss, Inc.

KEY WORDS: acute intermittent porphyria; AIP; HMBS; porphobilinogen deaminase; PBGD; Poland

INTRODUCTION

Acute intermittent porphyria (AIP, MIM# 176000) is an autosomal dominantly inherited disorder of heme biosynthesis resulting from an ~50% deficiency of hydroxymethylbilane synthase (HMBS) activity (EC 4.3.1.8; PBGD). This enzyme, also known as porphobilinogen deaminase (PBGD), catalyzes the head-to-tail condensation of four molecules of porphobilinogen (PBG) to form preuroporphyrinogen. Clinically, AIP manifests as intermittent attacks of neurovisceral dysfunction, including abdominal pain and neuropsychiatric symptoms. The

Received 1 October 2001; accepted 4 January 2002.

© 2002 WILEY-LISS, INC.
DOI: 10.1002/humu.9020

acute attacks are often precipitated by factors such as hormonal changes, stress, fasting, drugs and alcohol (Kappas et al., 1995). Diagnosis of AIP in patients can be readily made by measurement of the urinary porphyrin precursors, δ-aminolevulinic acid (ALA) and PBG, in combination with the PBG deaminase activity assay in the erythrocytes. However, only 10-20% of the individuals with a defective enzyme develop symptoms of AIP, while the majority of them remain asymptomatic throughout their lives. The accurate identification of presymptomatic AIPs so that they can be advised to avoid the attack-precipitating agents, is an essential element in the management of AIP. Recently, molecular analysis of the PBGD gene has been shown to be more efficient than enzymatic analysis in detecting latent AIPs who usually do not excrete excess amount of ALA and PBG in urine (Puy et al., 1997; Schuurmans et al., 2001).

The locus for this disorder has been mapped on chromosome 11q24.1-q24.2 (Namba et al., 1991). The length of the PBDG gene is ~10 kb, and the cDNA, encoded by 15 exons, is 1.4 kb, with a single open reading frame of 1038 bp (Grandchamp et al., 1987; Yoo et al., 1993; Genbank M95623). Two distinct promoters, located in the 5' flanking region and in intron 1, respectively, generate housekeeping (contains exon 1 and 3-15) and erythroid-specific (contains exon 2-15) transcripts by alternative splicing of exon 1 and 2 (Grandchamp et al., 1987). To date, a total of 172 different mutations have been identified in the PBGD gene, mostly from patients with the so called classical AIP in which both the housekeeping and erythroid-specific isozymes are affected (Human Gene Mutation Database, 2001). In <5% of the patients, the molecular defects are located in exon 1 of the PBDG gene which only affect the housekeeping enzyme – the so called variant AIP (Puy et al., 1998).

The present study was aimed at elucidating the molecular basis that underlines the clinical abnormalities seen in 20 Polish patients affected by AIP.

SUBJECTS AND METHODS

Patients

Twenty Polish patients from unrelated AIP families were investigated. The diagnosis of AIP was established based on typical clinical symptoms and elevated urinary δ-ALA and PBG concentrations. In 18 patients, erythrocyte PBG deaminase activities were below a range obtained from healthy individuals; whereas in one patient, the enzyme activity was within the normal range (Table 1). Peripheral blood samples from all 20 patients were collected after appropriate informed consent.

PCR amplification of genomic DNA

Genomic DNA was isolated from peripheral blood (EDTA-anticoagulated) by using the QIAampTM blood kit (Qiagen, Hilden, Germany). PCR amplification of all 15 exons of the PBGD gene was carried out by using primers described in Puy et al. (1997). PCR conditions were as follows: a 30 µl reaction mixture containing 0.2-1 µg of genomic DNA, 100 pmol of each primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200µM of each dNTPs was incubated at 94°C for 5 min. Twenty-microliters of a second reaction mixture containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2 and 3.5 U of "High-Fidelity" Taq polymerase (Roche Diagnostics, Basel, Switzerland) was then added ("hot start"). The reactions were carried out for 40 amplification cycles of 1 min at 94°C, 1 min at annealing temperatures for the respective exons (Puy et al., 1997) and 1 min elongation at 72°C.

DGGE analysis of the PBGD gene

Thirty-microliters of PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) in a "D GENE" System (BioRad, Hercules, CA, USA) according to the previously described conditions (Schneider-Yin et al., 2000). After electrophoresis, DGGE gels were stained with ethidium bromide and photographed under a UV transilluminator.

Direct sequencing

DNA fragments showing an abnormal DGGE pattern were purified by using the Qiaquick™ PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced with the BigDye Terminator cycle sequencing kit on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Restriction analysis

Ten-microliters of the PCR product of exon 5 from both patients' and control samples were digested with 10 units of restriction enzyme *Rsa* I (Roche Diagnostics, Basel, Switzerland) at 37°C for 2 hours. The digests were analyzed in a 3% agarose gel together with molecular weight markers. The agarose-gel was photographed under a UV-light after it was stained with ethidium bromide.

RESULTS AND DISCUSSION

We conducted a systematic analysis of the PBGD gene among AIP patients from Poland, a second of such studies in patients of Slavic origin (Rosipal et al., 1997). Among the 20 clinically diagnosed AIP patients, a total of 16 different mutations, including 7 novel mutations, were identified. As shown in Table 1, two of the seven newly identified mutations are frameshifts (315delT, 552delT) and one is a nonsense mutation (Y46X), which all would lead to abrupt mRNAs and therefore, truncated enzymes. Point mutation 771+2T>C at the donor splice site is likely to cause the skipping of exon 12 (see also Martinez di Montemuros et al., 2001). A single amino acid residue V93 deletion as the result of 278-280delTTG, would most probably inactivate the enzyme since V93 is involved in the formation of hydrophobic core based on the three-dimensional structure of PBG deaminase (Brownlie et al., 1994). The function of V93 in the enzyme was further demonstrated by expression of a mutant PBG deaminase V93F in *E. coli*, which exhibited a residual activity of <2% that of the wildtype enzyme (Chen et al., 1994).

Table 1. Biochemical and genetic abnormalities among Polish AIP patients

Patient /sex/age	PBG deaminase activity in RBC[a]	Urinary ALA (μmol/24 h)	Urinary PBG (μmol/24 h)	Exon or intron	Mutation position[c]	Amino acid change or other effect	Change in restriction enzyme sites
1/F/21	13.8	69.9	75.2	EX3	77G>A	R26H	*Aci* I
2/F/27	21.6	224.6	500.8	EX4	**168C>A**	**Y46X**	*Rsa* I
3/F/29	ND	260.3	404.0	EX5	**G181>T**	**D61Y**	*Rsa* I
4/F/50	38.1[b]	482.2	459.3	EX5	**G181>T**	**D61Y**	*Rsa* I
5/F/35	26.7[b]	67.9	438.9	EX7	**278-280delTTG**	**delV93**	-
6/F/29	28.1[b]	415.7	414.1	EX7	**315delT**	**stop at nt 401**	*Bsa* JI
7/F/34	10.8	225.7	248.8	EX7	G331G>A	G111R	*Nla* IV
8/F/32	25.0[b]	541.1	570.0	EX7	G331G>A	G111R	*Nla* IV
9/F/41	15.6	273.0	401.0	IVS7	344+1G>A	donor splice site	-
10/F/20	28.9[b]	270.2	706.0	EX9	C445C>T	R149X	-
11/F/45	14.6	200.6	348.3	EX10	G518G>A	R173Q	*Hpa* II
12/F/27	21.4	428.5	1143.0	EX10	G518G>A	R173Q	*Hpa* II
13/F/25	20.7	172.8	272.0	EX10	G518G>A	R173Q	*Hpa* II
14/F/53	23.3	311.3	493.0	EX10	**552delT**	**stop at nt 563**	*Hae* III
15/F/20	16.1	916.0	1098.4	EX12	673C>T	R225X	-
16/F/54	16.9	180.7	377.5	EX12	730-73delCT	stop at nt 747	*Hinf* I
17/F/41	16.9	508.7	616.1	IVS12	**771+2T>C**[d]	**donor splice site**	*Alw* NI
18/F/29	21.6	199.6	361.0	EX15	982-983delCA	stop at nt 1071	*Bsr* I
19/F/35	20.5	1860.0	1250.0	EX15	**992-1123del131**	**31 aa deletion**	-
20/F/36	23.9	260.1	540.0	EX15	1004G>A	G335D	-
normal	29.3-39.2	1.1-30.5	0.95-13.7	-	-	-	-

Novel mutations appear in **Boldface**.

ALA: aminolevulinic acid; PBG: porphobilinogen; ND: not determined; aa: amino acid.

[a] nmol uroporphyrin/ml RBC/h; [b] The activities were measured during the acute attacks. [c] The PBGD sequence is that of the cDNA of the Genbank accession no M95623; nucleotides are numbered with the A of the ATG initiation codon for the ubiquitous isoform as +1. [d]During review, this mutation was said to have been reported in an Italian patient with AIP, the data

having been presented at the 2001 World Congress on Iron Metabolism in Cairns, Australia ; abstract reportedly published as Martinez di Montemuros et al. (2001).

A 131-bp deletion (nucleotides 992-1123) was detected on one of PBGD alleles in a patient (patient No. 19) by both DGGE and sequence analyses of exon 15 (data reviewed but not shown). The mutant protein lacking 31 amino acids from the C-terminus - a part of the third enzyme domain, is likely the cause for enzyme deficiency and therefore the clinical outcome in this patient. Two apparently unrelated AIP patients (patients 3 and 4) shared a missense mutation G181>T which predicts the substitution of aspartic acid 61 to a tyrosine (D61Y; data reviewed but not shown). Asp61, a highly conserved amino acid residue, supposedly forms hydrogen bonds to other side chains and therefore plays a role in maintaining the overall stability of the enzyme (Brownlie et al., 1994). Another mutation involving this amino acid residue namely D61N (181G>A), has been found to be associated with the AIP phenotype in a British patient (Whately et al., 1999). All seven novel PBGD gene mutations described in this report were absent in over 100 alleles from non-porphyric individuals.

The majority of the 16 mutations identified among the Polish AIP patients have been previously reported in other European countries (Kauppinen et al., 1995; Puy et al, 1997, Whately et al., 1999, Martinez di Montemuros et al., 2000). Of the 9 known PBGD gene mutations, mutation 982-983delCA, however, has so far only been observed in a Japanese AIP patient, suggesting common origins of the PBGD gene mutations even among various races (Maeda et al., 2000). In addition, G111R was found to be the most frequent mutation in the Argentinean AIP population (De Siervi et al., 1999a, 2001).AIP is known to affect more females than males, as it is evident in the present study of all 20 patients being female. In a study of AIP in Argentina, a ratio of 7:3 was observed between female and male patients (De Siervi et al., 1999b). Recently, we analyzed the gender distribution among patients and latent carriers in a cohort of 21 DNA-diagnosed Swiss AIP families and were able to show that the difference in sex ratios between patients and carriers was statistically significant (Schuurmans et al., 2001). The pathogenic mechanism by which female gender predisposes to the disease expression is yet to be explored.This study has once again demonstrated that mutation analysis in the PBDG gene is more reliable than enzymatic assay (De Siervi et al., 2001). Although the enzyme activity values in 14 of the 20 AIP patients were clearly pathological, five patients showed a value that was near or even within the normal range. As we have experienced, the normal or near normal PBG deaminase activity in some AIP patients can occur when the measurement is performed during or immediately after acute attacks (Kostrzewska and Gregor, 1989; Minder, 1993). Indeed, molecular analysis is becoming the method of choice in the diagnosis of AIP.

ACKNOWLEDGMENT

We wish to thank Ms. H. Blickenstorfer for her excellent technical assistance. This work was supported by the Roche Research Foundation, grant 98-202.

REFERENCES

Brownlie P, Lambert R, Louie G, Jordan P, Blundell T, Warren M, Cooper J, Wood S. 1994. The three-dimensional structures of mutants of porphobilinogen deaminase: toward an understanding of the structural basis of acute intermittent porphyria. Protein Sci 3:1644-1650.

Chen C, Astrin K, Lee G, Anderson K, Desnick R. 1994. Acute intermittent porphyria: identification and expression of exonic mutations in the hydroxymethylbilane synthase gene. J Clin Invest 94:1927-1937.

De Siervi A, Rossetti M, Parera V, Astrin K, Aizencang G, Glass I, Batlle A, Desnick R. 1999a. Identification and characterization of hydroxymethylbilane synthase mutations causing acute intermittent porphyria; evidence for an ancestral founder of the common G111R mutation. Am J Med Genet 86:366-375.

De Siervi A, Rossetti M, Parera V, Mendez M, Varela L, Batlle A. 1999b. Acute intermittent porphyria: biochemical and clinical analysis in the Argentinean population. Clin Chim Acta 288:63-71.

De Siervi A, Varela L, Parera V, Batlle A, Rossetti M. 2001. Diagnosis of latent acute intermittent porphyria by genetic analysis. Ann Clin Biochem 38:149-152

Grandchamp B, de Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y. 1987. Tissue-specific expression of porphobilinogen deaminase. Two isozymes from a single gene. Eur J Biochem 162:105-110.

Kappas A, Sassa S, Galbraith RA, Nordmann Y. 1995. The porphyrias. in: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). Metabolic and Molecular Basis of Inherited Disease, 7th ed. McGraw-Hill, New York, pp. 2103-2160.

Kauppinen R, Mustajoki S, Pihlaja H, Peltonen L, Mustajoki P. 1995. Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. Hum Mol Genet 4: 215-222.

Kostrzewska E, Gregor A. 1989. Increased activity of porphobilinogen deaminase in erythrocytes during attacks of acute intermittent porphyria. Ann Clin Res 18:195-198.

Maeda N, Horie Y, Adachi K, Nanba E, Kawasaki H, Daimon M, Kudo Y, Kondo M. 2000. Two deletion mutations in the hydroxymethylbilane synthase gene in two unrelated Japanese patients with acute intermittent porphyria. J Hum Genet 45:263-2.

Martinez di Montemuros F, Di Pierro E, Fargion S, Biolcati G, Griso D, Macri A, Fiorelli G, Cappellini M. 2000. Molecular analysis of the hydroxymethlybilane synthase (HMBS) gene in Italian patients with acute intermittent porphyria: report of four novel mutations. Hum Mutat 15:480.

Martinez di Montemuros F, Di Pierro E, Bissolotti E, Tavazzi D, Danielli GM, Biolcati G, Cappellini MD, Fiorelli G. 2001. Five novel mutations in the hydroxymethylbilane synthase (HMBS) gene in Italian patients with acute intermittent porphyria (AIP). Blood Cells Molecules and Diseases 27:961-970. (N.B.: Abstract only; was unavailable to authors of present report)

Minder EI. 1993. Coproporphyrin isomers in acute-intermittent porphyria. Scand J Clin Lab Invest 53:87-90.

Namba H, Narahara K, Tsuji K, Yokoyama Y, Seino Y. 1991. Assignment of human PBG deaminase to 11q24.1-q24.2 by in situ hybridization and gene dosage studies. Cytogenet Cell Genet 57:105-108.

Puy H, Deybach JC, Lamoril J, Robreau AM, Da Silva V, Gouya L, Grandchamp B, Nordmann Y. 1997. Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. Am J Hum Genet 60:1373-1383.

Puy H, Gross U, Deybach JC, Robreau A, Frank M, Nordmann Y, Doss M. 1998. Exon 1 donor splice site mutations in the porphobilinogen deaminase gene in the non-erythroid variant form of acute intermittent porphyria. Hum Genet 103:570-575.

Rosipal R, Puy, H, Lamoril J, Martasek P, Nordmann Y, Deybach JC. 1997. Molecular analysis of porphobilinogen (PBG) deaminase gene mutations in acute intermittent porphyria: first study in patients of Slavic origin. Scand J Clin Invest 57:217-224.

Schneider-Yin X, Bogard C, Rüfenacht U, Puy H, Nordmann Y, Minder EI, Deybach J. 2000. Identification of a prevalent nonsense mutation (W283X) and two novel mutations in the porphobilinogen deaminase gene of Swiss patients with acute intermittent porphyria. Hum Hered 50: 247-250.

Schuurmans M, Schneider-Yin X, Rüfenacht U, Schnyder C, Minder CE, Puy H, Deybach JC, Minder EI. 2001. Influence of age and gender on the clinical expression of acute intermittent porphyria based on molecular study of porphobilinogen deaminase gene among Swiss patients. Mol Med 7:535-542.

Whately S, Woolf J, Elder G. 1999. Comparison of complementary and genomic DNA sequencing for the detection of mutations in the HMBS gene in British patients with acute intermittent porphyria: identification of 25 novel mutations. Hum Genet 104:505-510.

Yoo H, Warner C, Chen C, Desnick R. 1993. Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. Genomics 15:21-29.