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IDENTIFICATION AND CHARACTERIZATION OF *HMBS* GENE MUTATIONS IN SPANISH PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA

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Abstract – Acute intermittent porphyria (AIP), the most common acute hepatic porphyria, is an autosomal dominant disorder with low penetrance that results from a partial deficiency of hydroxymethylbilane synthase (HMBS), the third enzyme in the heme biosynthetic pathway. The disease is clinically characterized by acute neurovisceral attacks that are precipitated by several factors including certain drugs, steroid hormones, alcohol and fasting. Early diagnosis and counselling are essential to prevent attacks, being mutation analysis the most reliable method to identify asymptomatic carriers in AIP families. In this study we have investigated the molecular defect in 15 unrelated Spanish AIP patients. Mutation analysis of the *HMBS* gene revealed a total of fourteen mutations including six novel ones, two of them were on the same allele in one patient. The novel mutations were three missense (R26L, R173G and D178H), two frameshift (c.749_765dup and c.874insC) and one intronic deletion (IVS12+3_+11delAGGGCCTGT). RT-PCR and sequencing demonstrated that the intronic mutation caused abnormal splicing and exon 12 skipping. Prokaryotic expression of the novel missense mutations showed that only D178H had significant residual activity. These findings will facilitate the accurate identification of presymptomatic AIP carriers in these families and they further emphasize the molecular heterogeneity of AIP in Spain.

Key words: Porphyria, Acute intermittent porphyria, Hydroxymethylbilane synthase, Porphobilinogen deaminase, Mutation analysis, Splicing defect, Prokaryotic expression.

INTRODUCTION

Acute intermittent porphyria (AIP, MIM 176000) is the most common acute hepatic porphyria, and it is inherited as an autosomal dominant trait with incomplete penetrance (1).

Abbreviations: AIP, acute intermittent porphyria; **ALA**, 5-aminolevulinic acid; **ALAS1**, housekeeping 5-aminolevulinic acid synthase; **HMBS**, hydroxymethylbilane synthase; **NMD**, nonsense-mediated mRNA decay; **PBG**, porphobilinogen; **PCR**, polymerase chain reaction; **PTC**, premature termination codon; **RT**, reverse transcription

AIP results from a partial deficiency of hydroxymethylbilane synthase (HMBS; EC 4.3.1.8) also referred to as porphobilinogen deaminase. HMBS is the third enzyme in the heme biosynthetic pathway and it catalyzes the polymerization of four porphobilinogen (PBG) molecules to form hydroxymethylbilane.

The onset of clinical manifestations of AIP generally occurs during or after puberty, more frequently in women than in men and it is characterized by intermittent attacks that involve abdominal pain, vomiting, constipation, hypertension, tachycardia, peripheral neuropathy and psychiatric complaints (1). Acute attacks are precipitated by endogenous and exogenous

factors such as steroid hormones, certain drugs, alcohol and fasting all of which induce hepatic 5-aminolevulinic acid synthase (ALAS1), the first and rate-limiting enzyme in the heme biosynthesis (1). The increase in ALAS1 activity enhances the synthesis of porphyrin precursors, 5-aminolevulinic acid (ALA) and PBG. Thus partial HMBS deficiency in individuals heterozygous for mutations in the *HMBS* gene leads to insufficient conversion of the excess PBG into hydroxymethylbilane. This results in hepatic overproduction and massive urinary excretion of porphyrin precursors, along with the clinical symptoms of an acute attack of AIP (2, 16, 30). Therefore, early diagnosis and counselling regarding precipitating factors is essential to manage and prevent acute attacks in symptomatic patients and in latent heterozygous relatives.

The human *HMBS* gene has been mapped to the chromosomal region 11q24.1-q24.2, spanning a genomic interval of 10 kb. It contains 15 exons and it produces two mRNA transcribed from different promoters, that encode the housekeeping and the erythroid-specific isoforms of the enzyme (5, 24, 38). The housekeeping promoter is located in the 5' flanking region and its mRNA contains exon 1 joined to exons 3-15. The erythroid-specific promoter is in intron 1 and its mRNA contains exons 2-15 with the translation initiation codon lying in exon 3. The erythroid-specific enzyme contains 344 amino acids (~40 KD), whereas the housekeeping isoform (~42 KD) has an additional 17 residues at its N-terminus, of which eleven are encoded by exon 1.

Two subtypes of AIP have been described (1, 22, 25). In the classical form of AIP, the HMBS activity is reduced to about 50% of normal in all tissues, due to a mutation that affects both isoforms of the enzyme. In the non-erythroid variant AIP (2-5% of cases), the mutation only affects the housekeeping isoform and patients have normal HMBS activity in erythrocytes. In the asymptomatic phase of AIP, patients may excrete normal levels of porphyrin precursors and thus, the measurement of erythrocyte HMBS activity can be used to detect latent AIP individuals in family studies. Nevertheless, this approach is limited to families with classical AIP and in addition, there is a significant overlap between the heterozygote and the normal values observed (2). Therefore, when a disease-causing mutation has been identified in a proband, a molecular analysis is the most accurate method to identify asymptomatic gene carriers within AIP families (2, 30). About 300 mutations in the *HMBS* gene have been identified in AIP patients to date, indicative of the molecular heterogeneity of this porphyria (13) (see also the Human Gene Mutation Database at www.hgmd.cf.ac.uk/ac/index.php).

MATERIALS AND METHODS

Patients and biochemical determinations

Fifteen unrelated Spanish patients with AIP were studied here (Table 1). Fifty unrelated healthy individuals of Spanish origin were included as controls. The patients with AIP comprised fourteen females and one male residing in different geographical regions of Spain. All patients were diagnosed and were followed up clinically at the "12 de Octubre" Hospital (Madrid, Spain). The mean age of patients on their inclusion in the study was 47.5 ± 11.8 years (range: 28-69 years) and the mean age at the onset of the clinical manifestations was 27.3 ± 5.9 years (range: 18-37 years). The diagnosis of AIP was based on clinical symptoms, increased excretion of ALA and PBG in urine, as well as decreased erythrocyte HMBS activity. All patients were in remission at the time of the study and two patients were prophylactically treated with hematin at a dose of 3-4 mg/kg every 8-10 days (patients P3 and P6). These two patients had previously experienced more than 30 acute attacks, whereas the remaining patients had suffered between 1 and 7 attacks.

Urinary ALA and PBG concentrations were measured by the method of Mauzerall and Granick (20), while urinary excretion of porphyrins was determined by the method described by Westerlung et al (36). HMBS activity in erythrocytes was measured as described previously (29). Informed consent was obtained from each of the subjects before their inclusion in the study, and the project was approved by the Ethical Committee of the Hospital 12 de Octubre.

DNA analysis

Genomic DNA was extracted from EDTA-anticoagulated whole blood samples using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany). The *HMBS* gene was PCR amplified and sequenced using the primers described by Puy et al. (29) but without the GC clamp. All 15 exons with their flanking intron regions were amplified in five fragments (exon 1, exons 2-6, exons 5-9, exons 10-11 and exons 11-15). PCR products were purified using the GFX PCR DNA and gel Band Purification kit (GE Healthcare, Little Chalfont, UK), and sequenced in an ABI 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems). All mutations were confirmed on a second DNA sample. Nucleotides were numbered according to the cDNA sequence for the housekeeping isoform of HMBS (GenBank Accession Number NM_000190), in which the A of the ATG initiation codon was numbered as 1.

To determine the allelic distribution of the double variation identified (R173G and D178H), both alleles were separated by cloning using the pGEM-T Vector System (Promega Corporation. Madison, WI, USA), according to the manufacturer's protocol. Briefly, the PCR fragment containing the exon 10 from patient P7 was ligated into the

pGEM-T plasmid and transformed into *Escherichia coli* strain JM109. Twelve individual colonies were isolated and the cloned fragment was PCR amplified and sequenced.

RNA analysis

The effect of the newly identified intronic mutation on the mRNA splicing was studied by reverse transcription-PCR (RT-PCR) and sequencing. Leukocytes were isolated from EDTA-anticoagulated blood using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), and total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with an oligo(dT) primer and eAMV reverse transcriptase (Sigma-Aldrich, Inc. St. Louis, Missouri, USA). Subsequently, the full-length *HMBS* cDNA was amplified using the sense primer 5'-CCA CAC ACA GCC TAC TTT CCA AGC G-3' and the antisense primer 5'-TAG GCA CTG GAC AGC AGC AAC CCA-3'. The RT-PCR products were run on an agarose gel, each product was cut out of the gel, was purified using the GFX PCR DNA and gel Band Purification kit (GE Healthcare) and sequenced as described above.

Prokaryotic expression and characterization of the novel HMBS missense mutations

The novel missense mutations were expressed in *Escherichia coli* strain JM109 (Promega Corporation. Madison, WI, USA) using the pKK223-3 expression vector (Pharmacia Biotech Inc., Piscataway, NJ, USA). The human wild-type *HMBS* cDNA was obtained by RT-PCR on RNA isolated from the K562 cell line. RNA isolation and reverse transcription was performed as described above. The cDNA encoding the housekeeping HMBS was amplified using the primers S(ORF) and AS(ORF) (Table 2) and cloned into the *EcoRI-HindIII* sites of the pKK223-3 vector. This expression construct was designated pKK-HMBS-wt. Each of the missense mutations was introduced into the pKK-HMBS-wt by PCR based site-directed mutagenesis (6). PCR reactions were performed using the primers indicated in Table 2 and the pKK-HMBS-wt plasmid as template. For construction of pKK-HMBS-R26L, a fragment containing the desired mutation and the *KpnI* and *AvaIII* restriction sites was amplified with the S(26)/AS(E11) primer pair. The PCR product was then digested with the *KpnI* and *AvaIII* restriction endonucleases (New England Biolabs, Beverly, MA, USA), and purified using the GFX PCR DNA and gel Band Purification kit (GE Healthcare, Little Chalfont, UK). The purified *KpnI/AvaIII* fragment was ligated as a cassette into the corresponding sites in pKK-HMBS-wt and the resulting plasmid was transformed into *E. coli* JM109. A similar procedure was used to construct the expression vectors for the (R173G; D178H) allele (pKK-HMBS-R173G-D178H) and for the mutants R173G (pKK-HMBS-R173G) and D178H (pKK-HMBS-D178H), using the primer pairs S(E3)/AS(173/178), S(E3)/AS(173) and S(E3)/AS(178), respectively. The integrity of each expression construct was confirmed by sequencing.

Bacterial clones containing either the pKK223-3 vector or any of the pKK-HMBS expression constructs were grown to log phase and induced with 5 mM isopropylthiogalactoside (IPTG) for 3 hr. Cells were harvested by centrifugation and washed twice with PBS. The cell pellets were resuspended in 200 µL of lysis buffer (100 mM Tris-HCl buffer, pH 8.0, 0.1% Triton-X 100) and disrupted by sonication. The bacterial lysates were centrifuged and the supernatant was used as the source of the enzyme. HMBS activity was measured as published (29) and the specific activity was calculated as nmol uroporphyrinogen/hr/mg protein.

To study the thermal stability of the HMBS mutant D178H, the supernatants containing wild-type or mutant HMBS proteins were first diluted in 100 mM Tris-HCl buffer, pH 8.0, to a protein concentration of 1 mg/ml. The proteins were preincubated at 65ºC for between 0 and 180 min, after which they were placed on ice and the HMBS activity was measured as described above.

RESULTS

All patients in this study were diagnosed with classic AIP, displaying erythrocyte HMBS activities ranging from 41% to 67% of the control value (Table 1). The values of urinary precursors and porphyrins in these AIP patients in clinical remission ranged from: ALA (mg/l) = 8.1-43.7 (normal value: 0-5.5); PBG (mg/l) = 4.4-68.4 (normal value: 0-2); uroporphyrin (µg/24hr) = 14-1,268 (normal value: 15-50); coproporphyrin (µg/24hr) = 12-1,055 (normal value: 35-150).

In these probands, six novel and eight previously reported mutations were identified (Table 1). The novel mutations included three missense, two insertions and one splicing defect (Fig. 1). Interestingly, patient P7 was heterozygous for two novel mutations: R173G and D178H (Fig. 1a). To investigate whether these mutations were located on the same allele or on different alleles, the PCR product containing both mutations was cloned into the pGEM-T vector and 12 clones were sequenced. Two types of clones were found, either containing the wild type sequence or carrying both the R173G and D178H mutations, confirming that both changes reside in the same allele.

One of the two novel insertions was a 17 bp duplication of nucleotides c.749-c.765 (AAAGGGCCTTCCTGAGG) inserted between positions c.765 and c.766 in exon 12, designated c.749_765dup (Fig. 1b). This mutation changed amino acids 256-259 from HLEG to KGPS, and it introduced a premature translation stop site at codon 260. The other novel insertion occurred in exon 14 and was a single cytosine inserted at position c.874, designated c.874insC (Fig. 1b). This insertion caused a frameshift generating a premature translation termination signal 14 codons downstream, at the beginning of exon 15.

The novel splicing mutation was detected as a 9 bp deletion at the 5'end of intron 12 (positions +3 to +11), designated IVS12+3_+11delAGGGCCTGT (Fig. 1c). RT-PCR performed on RNA from the patient's

Table 1. *HMBS* gene mutations identified in this study

Patient (Sex)	**Onset age**	**HMBS activity** [a]	**Mutation** [b] **(Effect)**	**Location**	**Reference**
P1 (F)	22	51	c.77G>T (R26L)	Exon 3	This study
P2 (F)	32	46	c.101A>C (Q34P)	Exon 4	(7)
P3 (F)	35	63	c.340insT (frameshift and stop + 7)	Exon 7	(37)
P4 (F)	20	44	c.340insT (frameshift and stop + 7)	Exon 7	(37)
P5 (F)	22	55	c.499C>T (R167W)	Exon 10	(10)
P6 (F)	21	65	c.517C>T (R173W)	Exon 10	(21)
P7 (F)	32	65	c.517C>G; c.532G>C ([R173G; D178H])	Exon 10	This study
P8 (F)	18	58	IVS11-2delA (Exon 12 deletion)	Intron 11	(19)
P9 (F)	25	48	c.669_698del30 (ten amino acids deletion)	Exon 12	(11)
P10 (F)	29	41	c.669_698del30 (ten amino acids deletion)	Exon 12	(11)
P11 (M)	30	67	c.673C>T (R225X)	Exon 12	(15)
P12 (F)	33	45	c.749_765dup17 (p.256H> KGPSX)	Exon 12	This study
P13 (F)	27	53	IVS12+3_+11del9 (Exon 12 deletion)	Intron 12	This study
P14 (F)	37	47	c.874insC (frameshift and stop + 14)	Exon 14	This study
P15 (F)	26	57	c.913insC (frameshift and stop + 1)	Exon 15	(28)

[a] HMBS: Erythrocyte hydroxymethylbilane synthase activity expressed as the percentage of the mean value from 50 healthy individuals (mean ± SD: 98.5 ± 14.2 pmol uroporphyrinogen/hr/mg hemoglobin).
[b] Absence of these sequence deviations was confirmed in 50 unrelated healty (non-porphyric) individuals of Spanish origin. Reference sequences: GeneBank Accession numbers, M95623 (*HMBS* gene) and NM_000190 (*HMBS* cDNA).

Table 2. Primers used for expression studies

Primer	**Sequence (5'→3')**
S(ORF)	CCGGAATTCATGTCTGGTAACGGCAATGCG
AS(ORF)	CCCAAGCTTTTAATGGGCATCGTTAAGCTGC
S(26)	ATTCGCGTGGGTACC**t**CAAGAGC
AS(E11)	CCACAGCATACATGCATTCC
S(E3)	ATTCGCGTGGGTACCCGCAAGAGC
AS(173)	TGTTGCCAGGATGATGGCACTGAACTCCTGCTGCTCGTCCAGCTTCCGAAGCC**c**GGTGTTG
AS(178)	TGTTGCCAGGATGATGGCACTGAACTCCTGCTGCTCGT**g**CAGCTTCCGAAGCCGGGTGTTG
AS(173/178)	TGTTGCCAGGATGATGGCACTGAACTCCTGCTGCTCGT**g**CAGCTTCCGAAGCC**c**GGTGTTG

The nucleotides underlined indicate the restriction sites for the endonucleases *EcoRI* (in S(ORF)), *HindIII* (in AS(ORF)), *KpnI* (in S(26) and S(E3)), *AvaIIII* (in AS(E11)), and *BstXI* (in AS(173), AS(178) and AS(173/178)). In the primers used for mutagenesis, the mutated base is indicated by bold lower case letters. S: sense; AS: antisense.

leukocytes gave two products, one of normal size and another smaller one. Sequencing of these products revealed that the normal sized product had the wild type sequence, whereas the entire 120 bp exon 12 was deleted in the smaller product and exon 11 was joined precisely to exon 13 (Fig. 1d). This exon skipping maintains the reading frame and predicts the synthesis of a protein that is missing 40 amino acids (residues 218 to 257).

The novel missense mutations were expressed in *E. coli* to study the functional consequences of these alterations. As shown in Table 3, only the D178H mutation resulted in an enzyme with significant residual activity (63%). However stability studies revealed that the D178H mutant protein was less thermostable than the wild-type enzyme (Fig. 2).

DISCUSSION

Among 15 unrelated Spanish patients, 14 different mutations were identified of which 6 were novel mutations: three missense mutations; one intronic deletion; and two frameshift mutations. The three novel missense mutations (R26L, R173G and D178H) affect amino acids that are highly conserved throughout evolution (4). Two of these mutations (R173G and D178H) coexisted on one allele and prokaryotic expression studies demonstrated that this allele expressed an inactive polypeptide. However, the expression of the individual mutation in *E. coli* showed that they each had a different effect on the protein: the R173G mutation inactivated the enzyme while the D178H mutation resulted in an enzyme with 63% of normal activity and decreased thermostability. The R26L mutation also caused a dramatic decrease in the enzyme activity expressed in *E. coli* and, like the R173G mutation, it affected an invariant residue involved in catalysis (4, 14, 17). When other substitutions of the same residues (R26C, R26H, R173W and R173Q) have been studied in prokaryotic or eukaryotic expression systems, the mutant proteins also exhibited very low or no residual activity (3, 8, 23, 26, 32). The residue D178 forms a salt bridge to R201, a residue located in a surface loop between an alpha-helix and a beta-sheet (4, 33). Interestingly, another missense mutation at the same codon, D178N, resulted in a thermo labile polypeptide with 81% of residual activity when was expressed in *E. coli* (35).

The two novel frameshift mutations c.749_765dup and c.874insC, introduce premature termination codons (PTCs) in exon 12 and exon 15, respectively. Accordingly, the

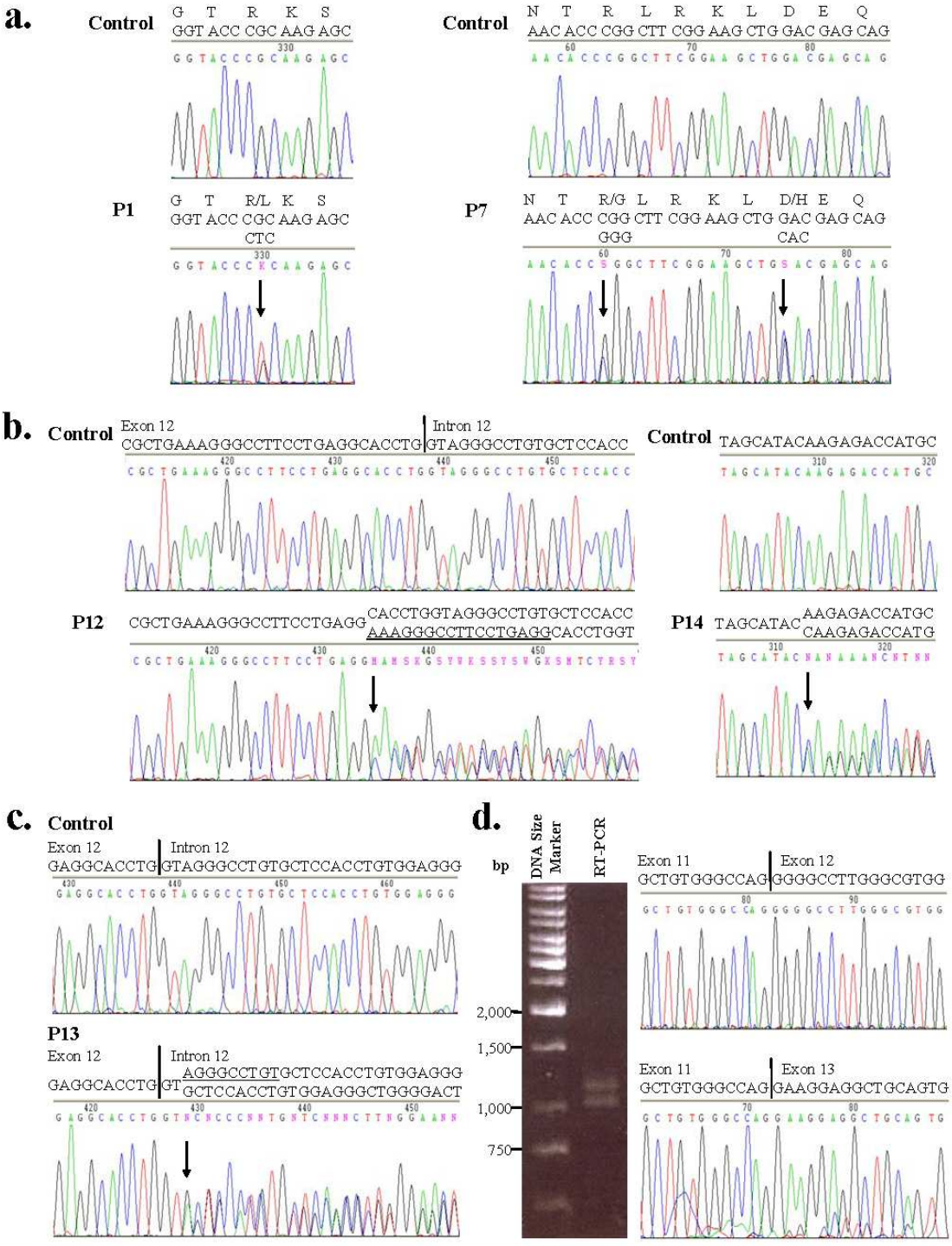

Figure 1. Novel mutations identified in the *HMBS* gene. **(a, b, c)** electropherograms showing the relevant part of the sequence in a control individual (top) and in the affected patients (bottom). **(a)** Left panel: mutation R26L identified in exon 3 from patient P1. Right panel: mutations R173G and D178H identified in exon 10 from patient P7. The position of the mutant nucleotides are indicated by arrows and the amino acid sequences are shown. **(b)** Left panel: mutation c.749_765dup identified in exon 12 from patient P12. The arrow indicates the beginning of the insertion and the nucleotides inserted are underlined. Right panel: mutation c.874insC identified in exon 14 from patient P14. The arrow indicates the site of the insertion. **(c)** Mutation IVS12+3_+11delAGGGCCTGT identified in patient P13. The arrow indicates the beginning of the deletion and the nucleotides deleted in the mutant allele are underlined in the sequence of the normal allele. **(d)** Left panel: agarose-gel electrophoresis of the *HMBS* RT-PCR products from patient P13. Right panel: partial sequence electropherograms of the two RT-PCR products from the patient, a normal size product (top) and a smaller product in which exon 12 has been skipped (bottom).

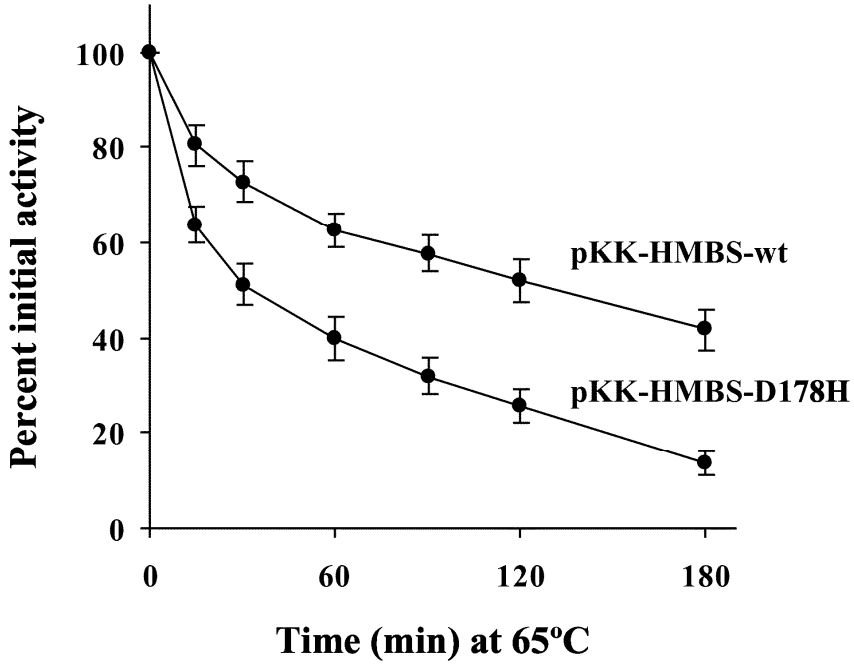

Figure 2. Thermostability of wild-type HMBS and mutant D178H. HMBS activity is expressed as the percentage of the initial activity (Mean ± SD from four independent experiments).

Table 3. Prokaryotic expression of *HMBS* missense mutations

Construct	**HMBS specific activity (nmol uroporphyrinogen/hr/mg)**	**Residual Activity (%)**
	Mean ± SD (Range)	
pKK223-3	1.47 ± 0.04 (1.41-1.51)	0
pKK-HMBS-wt	219.42 ± 11.34 (206.09-233.80)	100
pKK-HMBS-R26L	1.47 ± 0.03 (1.42-1.50)	0
pKK-HMBS-R173G-D178H	1.48 ± 0.03 (1.44-1.51)	0
pKK-HMBS-R173G	1.48 ± 0.02 (1.45-1.51)	0
pKK-HMBS-D178H	138.59 ± 15.90 (115.73-152.09)	63

HMBS specific activity (SA) was determined in four independent experiments. Residual activity was calculated by dividing 100 x ($SA-SA_{pKK223-3}$) by ($SA_{pKKHMBS-wt} - SA_{pKK223-3}$).

transcript produced from the c.749_765dup allele is most likely degraded by nonsense-mediated mRNA decay (NMD) (12, 18). By contrast, since the PTC introduced by the c.874insC mutation arises in the last exon, the mutant transcript is unlikely to be subject to NMD, predicting the synthesis of a truncated polypeptide (18).

The novel intronic deletion occurred at the 5' end of intron 12 (IVS12+3_+11delAGGGCCTGT) and it changed the donor splice site from CTGgtaggg to CTGgtgctc (consensus sequence: (A/C)AGgt(a/g)agt). RT-PCR experiments demonstrated that this deletion disrupts the donor splice site, causing the skipping of exon 12. Other mutations that caused exon 12 skipping have also been described previously and this deletion produces an inactive protein (9, 13, 27).

A total of 16 *HMBS* mutations have been previously identified in Spain, among which the most frequent are R173W and c.669_698del30 (11, 31, 34). Interestingly, the former is prevalent in Catalonia (Northeast Spain), while the latter is common in Murcia (Southeast Spain) and is due to a founder effect (11, 34). In the present study of Spanish patients from 15 unrelated AIP families, we identified 8 known and 6 novel mutations, of which two were in the same allele. Of the 8 mutations already reported, four (c.340insT, R167W, R173W and c.669_698del30) had previously been found in the Spanish population (11, 31, 34). The mutations identified in this study will allow the accurate diagnosis of asymptomatic heterozygotes in these Spanish families and further emphasize the molecular heterogeneity of AIP in Spain.

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REFERENCES

1. Anderson, K.E., Sassa, S., Bishop, D.F. and Desnick R.J., Disorders of heme biosynthesis: X-linked sideroblastic anemia and the porphyrias. In: *The Metabolic and Molecular Bases of Inherited Disease*, Scriver, C.S., Beaudet. A.L., Sly, W.S., Valle, D. (eds.), McGraw-Hill, New York, 2001, pp. 2991-3062.

2. Anderson, K.E., Bloomer, J.R., Bonkovsky, H.L., Kushner, J.P., Pierach, C.A., Pimstone, N.R., and Desnick, R.J., Recomendations for the diagnosis and treatment of the acute porphyrias. *Ann. Intern. Med*. 2005, **142**: 439-450.

3. Brons-Poulsen, J., Christiansen, L., Petersen, N.E., Horder, M., and Kristiansen, K., Characterization of two isoalleles and three mutations in both isoforms of purified recombinant human porphobilinogen deaminase. *Scand. J. Clin. Lab. Invest.* 2005, **65**: 93-106.

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

5. Chretien, S., Dubart, A., Beaupain, D., Raich, N., Grandchamp, B., Rosa, J., Goossens, M., and Romeo, P.H., Alternative transcription and splicing of human porphobilinogen deaminase gene result either in tissue-specific or in housekeeping expression. *Proc. Natl. Acad. Sci. USA.* 1988, **85**: 6-10.

6. Cormack, B., Mutagenesis by the polymerase chain reaction. In: *Current Protocols in Molecular Biology*, Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.), John Wiley and Sons, New York, 1999, Vol. 1. 8.5.1-9.

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

8. Delfau, M.H., Picat, C., de Rooij, F.W., Hamer, K., Bogard, M., Wilson, J.H.P., and Deybach, J.C., Two different point G to A mutations in exon 10 of the porphobilinogen deaminase gene are responsible for acute intermittent porphyria. *J. Clin. Invest.*1990, **86**: 1511-1516.

9. Grandchamp, B., Picat, C., de Rooij, F., Beaumont, C., Wilson, P., Deybach, J.C., and Nordmann, Y., A point mutation G>A in exon 12 of the porphobilinogen deaminase gene results in exon skipping and is responsible for acute intermittent porphyria. *Nucleic Acids Res.* 1989, **17**: 6637-6649.

10. Gu, X.F., De Rooij, F., Voortman, G., Te Velde, K., Nordmann, Y., and Grandchamp, B., High frequency of mutations in exon 10 of the porphobilinogen deaminase gene in patients with a CRIM-positive subtype of acute intermittent porphyria. *Am. J. Hum. Genet.* 1992, **51**: 660-665.

11. Guillen-Navarro, E., Carbonell, P., Glover, G., Sanchez-Solis, M., and Fernandez-Barreiro, A., Novel HMBS founder mutation and significant intronic polymorphism in Spanish patients with acute intermittent porphyria. *Ann. Hum. Genet.* 2004, **68**: 509-514.

12. Hentze, M.W., and Kulozik, A.E., A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* 1999, **96**: 307-10.

13. Hrdinka, M., Puy, H., and Martasek, P., May 2006 update in porphobilinogen deaminase gene polymorphisms and mutations causing acute intermittent porphyria. Comparison with the situation in Slavic population. *Physiol. Res.* 2006, **55**(Suppl 2): S119-S136.

14. Jordan, P.M., and Woodcock, S.C., Mutagenesis of arginine residues in the catalytic cleft of *Escherichia coli* porphobilinogen deaminase that affects dipyrromethane cofactor assembly and tetrapyrrole chain initiation and elongation. *Biochem. J.* 1991, **280**: 445-449.

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

16. Kauppinen, R., Porphyrias. *Lancet*. 2005**, 365**: 241-252.

17. Lander, M., Pitt, A.R., Alefounder, P.R., Bardy, D., Abell, C., and Battersby, A.R., Studies on the mechanism of hydroxymethylbilane synthase concerning the role of arginine residues in substrate binding. *Biochem. J.* 1991, **275**: 447-452.
18. Maquat, L.E., Nonsense-mediated mRNA decay: Splicing, translation and mRNP dynamics. *Nat. Rev. Mol. Cell Biol.* 2004, **5**: 89-99.
19. Martinez di Montemuros, F., Di Pierro, E., Biolcati, G., Rocchi, E., Bissolotti, E., Tavazzi, D., Fiorelli, G., and Cappellini, M.D., Acute intermittent porphyria: Heterogeneity of mutations in the hydroxymethylbilane synthase gene in Italy. *Blood Cells Mol. Dis.* 2001. **27**: 961-970.
20. Mauzerall, D., and Granick, S., The occurrence and determination of delta-aminolevulinic acid and porphobilinogen in urine. *J. Biol. Chem.* 1956, **219**:435-446.
21. Mgone, C.S., Lanyon, W.G., Moore, M.R., Louie, G.V., and Connor, J.M., Identification of five novel mutations in the porphobilinogen deaminase gene. *Hum. Mol. Genet.* 1994, **3**: 809-811.
22. Mustajoki, P., Normal erythrocyte uroporphyrinogen I synthase in a kindred with acute intermittent porphyria. *Ann. Intern. Med*. 1981, **95**: 162-166.
23. Mustajoki, S., Laine, M., Lahtela, M., Mustajoki, P., Peltonen, L., and Kauppinen, R., Acute intermittent porphyria: Expression of mutant and wild-type porphobilinogen deaminase in COS-1 cells. *Mol. Med.* 2000, **6**: 670-679.
24. Namba, H., Narahara, K., Tsuji, K., Yokohama, Y., and Senio, Y., Assignment of human porphobilinogen deaminase to 11q24.1 q24.2 by in situ hybridization and gene dosage studies. *Cytogenet. Cell Genet.* 1991, **57**: 105-108.
25. Nordmann, Y., and Puy, H., Human hereditary hepatic porphyrias. *Clin. Chim. Acta*. 2002, **325**: 17-37.
26. Ong, P.M.L., Lanyon, W.G., Graham, G., Hift, R.J., Halkett, J., Moore, M.R., and Connor, J.M., Acute intermittent porphyria: The *in vitro* expression of mutant hydroxymethylbilane synthase. *Mol. Cell. Probes* 1997, **11**: 293-296.
27. Pischik, E., Mehtälä, S., and Kauppinen, R., Nine mutations including three novel mutations among Russian patients with acute intermittent porphyria. *Hum. Mutat.* 2005, **26**: 496.
28. Puy, H., Deybach, J.C., Lamoril, J., Robreau, A.M., and Nordmann, Y., Detection of four novel mutations in the porphobilinogen deaminase gene in French Caucasian patients with acute intermittent porphyria. *Hum. Hered.* 1996, **46**: 177-180.
29. Puy, H., Deybach, J.C., Lamoril, J., Robreau, A.M., Da Silva, V., Gouya, L., Grandchamp, B., and Nordmann, Y., Molecular Epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. *Am. J. Hum. Genet*. 1997, **60**:1373-1383.
30. Sassa, S., Modern diagnosis and management of the porphyrias. *Br. J. Hematol*. 2006, **135**: 281-292.
31. Solis, C., Lopez-Echaniz, I., Sefarty-Graneda, D., Astrin, K.H., and Desnick, R.J., Identification and expression of mutations in the hydroxymethylbilane synthase gene causing acute intermittent porphyria (AIP). *Mol. Med.* 1999, **5**: 664-671.
32. Solis, C., Martinez-Bermejo, A., Naidich, T.P., Kaufmann, W.E., Astrin, K.H., Bishop, D.F., and Desnick, R.J., Acute intermittent porphyria. Studies of the severe homozygous dominant disease provides insights into the neurologic attacks in acute porphyrias. *Arch. Neurol.* 2004, **61**: 1764-1770.
33. Song, G., Li, Y., Cheng, C., Zhao, Y., Gao, A., Zhang, R., Joachimiak, A., Shaw, N., Liu, Z.J., Structural insight into acute intermittent porphyria. *FASEB J.* 2009, **23**: 396-404.
34. To-Figueras, J., Badenas, C., Carrera, C., Muñoz, C., Mila, M., Lecha, M., and Herrero, C., Genetic and biochemical characterization of 16 acute intermittent porphyria cases with a high prevalence of the R173W mutation. *J. Inherit. Metab. Dis.* 2006, **29**: 580-585.
35. Ulbrichova, D., Schneider-Yin, X., Mamet, R., Saudek, V., Martasek, P., Minder, E.I., and Schoenfeld, N., Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria. *Blood Cells Mol. Dis.* 2009, **42**:167-173.
36. Westerlund, J., Pudek, M., and Schreiber, W.E., A rapid and accurate spectrofluorometric method for quantification and screening of urinary porphyrins. *Clin. Chem.* 1988, **34**: 345-351.
37. Whatley, S.D., Woolf, J.R., and Elder, G.H., 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.* 1999, **104**: 505-510.
38. Yoo, H,W., Warner, C.A., Chen, C.H., and Desnick, R.J., Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. *Genomics*. 1993, **15**: 21-29.