

# Identification and Expression of Mutations in the Hydroxymethylbilane Synthase Gene Causing Acute Intermittent Porphyria (AIP)

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## Abstract

**Background:** Acute intermittent porphyria (AIP), an autosomal dominant inborn error, results from the half-normal activity of the heme biosynthetic enzyme hydroxymethylbilane synthase (EC 4.3.1.8; HMB-synthase). This disease is characterized by acute, life-threatening neurologic attacks that are precipitated by various drugs, hormones, and other factors. The enzymatic and/or biochemical diagnosis of AIP heterozygotes is problematic; therefore, efforts have focused on the identification of HMB-synthase mutations so that heterozygotes can be identified and educated to avoid the precipitating factors. In Spain, the occurrence of AIP has been reported, but the nature of the HMB-synthase mutations causing AIP in Spanish families has not been investigated. Molecular analysis was therefore undertaken in nine unrelated Spanish AIP patients.

**Materials and Methods:** Genomic DNA was isolated from affected probands and family members of nine unrelated Spanish families with AIP. The HMB-synthase

gene was amplified by long-range PCR and the nucleotide sequence of each exon was determined by cycle sequencing.

**Results:** Three new mutations, a missense, M212V; a single base insertion, g4715insT; and a deletion/insertion, g7902ACT→G, as well as five previously reported mutations (G111R, R116W, R149X, R167W, and R173W) were detected in the Spanish probands. Expression of the novel missense mutation M212V in *E. coli* revealed that the mutation was causative, having <2% residual activity.

**Conclusions:** These studies identified the first mutations in the HMB-synthase gene causing AIP in Spanish patients. Three of the mutations were novel, while five previously reported lesions were found in six Spanish families. These findings enable accurate identification and counseling of presymptomatic carriers in these nine unrelated Spanish AIP families and further demonstrate the genetic heterogeneity of mutations causing AIP.

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## Introduction

Acute intermittent porphyria (AIP), an autosomal dominant inborn error of metabolism, results from the half-normal activity of hydroxymethylbilane synthase (EC 4.3.1.8; HMB-synthase), the

third enzyme in the heme biosynthetic pathway (1,2). This enzyme, also known as porphobilinogen deaminase, catalyzes the head-to-tail condensation of four molecules of porphobilinogen (PBG) to form the linear tetrapyrrole, hydroxymethylbilane. Clinical onset of the disease typically occurs during or after puberty and is characterized by acute, life-threatening attacks of neurological dysfunction, including abdominal pain and other gastrointestinal complaints, hy-

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pertension, tachycardia, and various peripheral and central nervous system manifestations. Expression of the disease is highly variable, determined in part by environmental, metabolic, and hormonal factors that induce hepatic  $\delta$ -aminolevulinic acid synthase activity, the first and rate-limiting enzyme of heme biosynthesis, thereby increasing the production of the porphyrin precursors  $\delta$ -aminolevulinic acid (ALA) and PBG (1,2). The half-normal hepatic HMB-synthase activity in AIP patients is insufficient to prevent precipitation of acute attacks of the disease. Thus, diagnosis of AIP heterozygotes is crucial as the primary form of medical management in the avoidance of specific precipitating factors.

Two major subtypes of AIP have been delineated. In classical AIP, the HMB-synthase activity is half-normal in all cells and tissues, whereas in variant AIP (representing ~5% of AIP families), the non-erythroid tissues have half-normal activity, but the enzyme in erythrocytes is expressed at normal levels (1,3). Symptomatic heterozygotes with classical or variant AIP, which excrete increased levels of the porphyrin precursors ALA and PBG, can be identified easily, provided that the diagnosis is considered. However, the biochemical diagnosis of asymptomatic heterozygotes, which usually have normal levels of urinary ALA and PBG, has been problematic by enzyme assay, primarily because of the significant overlap between high heterozygote and low normal values (4,5). Moreover, identification of asymptomatic heterozygotes for variant AIP is not feasible since they have normal erythrocyte enzymatic activities. In view of the difficulties with biochemical diagnosis, investigators have turned to molecular techniques to identify specific mutations in the HMB-synthase gene for accurate diagnosis of affected members in AIP families.

For the precise diagnosis of AIP, efforts have been directed toward identifying the specific mutation in the HMB-synthase gene in each AIP family. The entire 10 kb HMB-synthase gene has been sequenced, including the 5' regulatory, 3' untranslated, and intronic regions (6). The gene contains 15 exons and 2 distinct promoters that generate housekeeping and erythroid-specific transcripts by alternative splicing (7,8). The housekeeping promoter is in the 5' flanking region and its transcript contains exons 1 and 3 through 15, while the erythroid-specific promoter is in intron 1 and its transcript is encoded by exons 2 through 15.

Most of the mutations causing AIP have

been private (i.e., unique to one family) or were found only in a few unrelated families, which emphasizes the molecular heterogeneity of the mutations causing this disease. Although no common mutations have been identified, several mutations are common in specific populations, their high frequencies presumably due to common founders as demonstrated by haplotype analysis for Swedish and Argentinean patients (9–11). Among these are mutations W198X, R116W, and G111R, which have been detected in a large number of Swedish, Dutch, and Argentinean probands, respectively (10–12). To date, mutations in the HMB-synthase gene causing AIP have not been reported in Spanish patients. In this report, long-range polymerase chain reaction (PCR) and cycle sequencing were employed to identify the HMB-synthase mutations in nine Spanish AIP families. Three new and five previously reported mutations were identified. The newly identified missense mutation was expressed in *E. coli* and shown to be causative, having low residual activity, while four of the five previously reported mutations occurred at CpG dinucleotides.

## Materials and Methods

### *Patient Specimens and Biochemical Assays*

Peripheral blood samples were collected with informed consent from nine unrelated Spanish AIP patients and their relatives. As shown in Table 1, the diagnosis of AIP was confirmed in each proband by determining their erythroid HMB-synthase activity and/or levels of urinary ALA, PBG, and porphyrins (13,14). Genomic DNA was extracted from peripheral blood using the Puregene DNA isolation kit (Gentra Systems, Minneapolis MN).

### *Long-Range PCR*

The entire HMB-synthase gene (10 kb) was amplified from genomic DNA in two fragments: fragment 1, including the promoter region through intron 3 (4.5 kb PCR product), and fragment 2, containing exon 2 through exon 15 (5.5 kb PCR product) as previously described (11). Briefly, each fragment was amplified using the GeneAmp XL PCR Kit (Perkin Elmer, Foster City CA) as follows: an initial denaturation was performed at 94°C for 1 min, and then amplification was carried out in a PCR Minicycler (M.J. Research, Watertown, MA) of both fragments 1 and

Table 1. Biochemical characterization of the Spanish AIP probands

Proband/sex	Age at diagnosis (years)	Erythrocyte HMB-synthase activity (nmol/hr/ml packed erythrocytes)	Urine		
			Porphyrins <sup>a</sup> (μg/24 hr)	ALA (mg/24 hr)	PBG (mg/24 hr)
1/F	29	20.6	9860	ND	ND
2/F	30	14.8	156 <sup>c</sup>	8.1 <sup>b</sup>	4.6 <sup>b</sup>
3/F	26	16.0	864	19.7	10.2
4/F	28	15.0	1390	14.0	9.0
5/F	26	19.6	85 <sup>b</sup>	4.3 <sup>b</sup>	0.4 <sup>b</sup>
6/F	23	25.8	2840	87.3	63.0
7/M	27	23.1	ND	ND	ND
8/F	34	23.0	479	12.0	9.0
9/F	27	22.8	ND	32.5	56.0
Normal	—	34.5 ± 4.7 <sup>c</sup>	<200	<7	<2

ND, not determined.

<sup>a</sup>Total porphyrins in urine (13).

<sup>b</sup>Urines were not collected from patient during an acute attack.

<sup>c</sup>Mean ± 1 SD.

2 in a single or separate reaction(s). The first 16 cycles were performed with denaturation at 94°C for 30 sec and annealing and extension at 67°C for 5 min. The second 12 cycles were carried out with denaturation at 94°C for 30 sec, and annealing and extension at 67°C for 5 min. The second 12 cycles were carried out with denaturation at 94°C for 30 sec, and annealing and extension at 67°C for 5 min and 15 sec, with 15-sec increments between each additional cycle. The final extension step was performed at 72°C for 10 min. A portion of each PCR product was analyzed by agarose gel electrophoresis to determine whether the long-range reactions were successful and to identify any gross gene rearrangements. Each PCR product was purified with the QIAquick PCR Purification Kit (QIAGEN, Santa Clara, CA).

#### Sequencing Reactions

Each exon and the flanking intronic regions were sequenced using the Amplicycle™ Sequencing Kit (Perkin Elmer) according to the manufacturer's instructions using the sense and antisense primers listed in ref. 11 and the following modifications. The cycle sequencing reaction was performed (30 cycles) with a denaturation

step of 30 sec at 95°C, annealing for 30 sec at 60°C, and extension for 60 sec at 72°C. After a denaturation step of 3 min at 94°C, a portion of the PCR reaction was loaded in a 4% acrylamide gel containing 7 M urea. The gels were dried, placed against Kodak X-OMAT film for 24 to 48 hr and then developed.

#### Prokaryotic Expression of HMB-Synthase Mutations

The normal and M212V HMB-synthase alleles were individually expressed in *E. coli* using the pKK223-2 vector (Pharmacia Biotech., Piscataway, NJ) as described previously (11,15). To introduce the mutations into the normal pKK-HMB-synthase (pKK-HMBS) expression construct, site-directed mutagenesis was performed (16) with the mutation being introduced into the construct by PCR amplification. For the M212V mutation, sense and antisense primers CS52 (5'-GAGTTCAGTGCCATCATCCTGGCAACAGCTGGCCTGCAGCGCATGGGCTGGCACAACCGGGTTGGGCAGATCCTGCACCCTGAGGAATGCGTGTAT-3') and CS53 (5'-GCTATCTGAGCCGTCTAGACTCCAGACTCCT-3') were used to generate a 326 bp amplification product. The reaction was performed in a final volume of 100 μl containing 10× Tris-HCl buffer, pH 8.3 (10 μl), 1 mM of MgCl<sub>2</sub>, 200 μM of

**Table 2. Mutations in Spanish AIP probands**

Proband	Exon	Mutation	Nucleotide change	Amino acid change	References
1	7	G111R	331G→A	111Gly→Arg	20
2	7	g4715insT	Insertion of T after g4715	114C→LQAGKPSX	This report
3	8	R116W	346C→T	116Arg→Trp	12
4	9	R149X	445C→T	149Arg→Term	21
5	10	R167W	499C→T	167Arg→Trp	22,23
6	10	R173W	517C→T	173Arg→Trp	24
7	10	R173W	517C→T	173Arg→Trp	24
8	11	M212V	634A→G	212Met→Val	This report
9	14	g7902ACT→G	<sup>a</sup>	<sup>a</sup>	This report

<sup>a</sup>Deletion of nt ACT at g7902-g7904 and insertion of a G resulted in a frameshift with 10 new amino acid residues (GRSLESRLRX) starting at codon 279 and termination after codon 289.

each dNTP, 1 ng of wild-type pKK-HMBS, 25 pmol of each primer, and 2.5 U of *Taq* polymerase (Life Technologies). PCR amplification was performed with an initial denaturation at 94°C for 4 min and then 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 30 sec. The amplification product was digested with *Xba*I and *Bst*XI after purification with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) and was ligated as a cassette into the corresponding sites in pKK-HMBS, generating the mutant construct pKK-HMBS-M212V. After transfection, bacterial growth, and isopropylthiogalactoside (IPTG) induction, the expressed HMB-synthase activity was assayed as previously described (15,17).

## Results

Molecular analysis of the HMB-synthase gene was performed in nine unrelated Spanish AIP probands. Three new and five previously reported mutations were identified by long-range PCR and cycle sequencing (Table 2). The three novel mutations included a missense mutation (M212V), a single base insertion (g4715insT), and a deletion/insertion mutation (g7902ACT→G).

The M212V mutation resulted from an A-to-G transition of genomic nt g7104 in exon 11 predicting the substitution of a neutral hydrophobic valine for a neutral hydrophobic methionine (Fig. 1A). This substitution was isofunctional and occurred in a region that was not

phylogenetically conserved. To further characterize this base substitution, a pKK-HMBS expression vector containing M212V was constructed, and expressed in *E. coli*, and the enzymatic activity of the mutant protein was determined. As shown in Table 3, the residual enzymatic activity of the mutant protein was 1.7% of the mean enzymatic activity expressed by the normal allele, indicating that the base substitution was indeed a causative missense mutation.

The second novel mutation was the insertion of a single thymidine at genomic nt 4715 (cDNA nt 340) in exon 7, which resulted in a frameshift that predicted substitution of seven amino acids in codons 114 to 120 (114CKRENPH→114LQAGKPSX) and chain termination (codon 121), thereby deleting the last two-thirds of the 361 amino acids of the monomeric housekeeping isoenzyme (Fig. 1B). The third novel mutation was a deletion/insertion in exon 14 at genomic nt g7902 (cDNA nt 835), involving the deletion of the three nucleotides ACT, and their replacement by a single G (Fig. 1C). This deletion/insertion resulted in a frameshift and predicted the replacement of the next nine amino acids in codons 279 to 289 (279TGGVWVSLDGS by 279GRSLESRRLRX) prior to termination at codon 289, thereby deleting the last 73 amino acids of the housekeeping isoenzyme.

In the remaining six Spanish AIP families, each had a previously reported mutation. These included one nonsense and four missense muta-



**Table 3. Expression of HMB-synthase mutation M212V in *E. coli***

Construct	HMB-synthase activity (U/mg/hr)		Percent of mean normal expressed activity
	Mean <sup>a</sup>	Range	
JM109	0.2	0.1–0.3	0.0
pKK-HMBS	75.0	67.8–83.2	100.0
pKK-HMBS-M212V	1.3	1.1–1.7	1.7

<sup>a</sup>Mean and range based on results of three independent experiments.

tions: G111R in exon 7, R116W in exon 8, R149X in exon 9, and both R167W and R173W in exon 10. The latter mutation was identified in two unrelated Spanish AIP probands.

## Discussion

This is the first report of HMB-synthase mutations that cause AIP in the Spanish population. Using a long-range PCR strategy, all exons and their adjacent intronic boundaries were amplified and sequenced. Three novel lesions, including a missense mutation, an insertion, and a deletion/insertion, were identified in three unrelated families. The novel M212V mutation substituted an isofunctional valine for a methionine in a phylogenetically nonconserved position. However, prokaryotic expression of the M212V protein revealed that the enzyme was markedly impaired, having <2% of the expressed wild-type enzyme. These findings confirm that the base substitution was not a benign polymorphism, but was the causative mutation. The novel g4715insT mutation in exon 7 joins several other small insertions (1 to 8 bases) and small deletions (1 to 2 bases) that cause frameshift mutations and early truncation of the enzyme polypeptide [Human Gene Mutation Database; <http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html>]. In this case, the g4715insT lesion resulted in the replacement of amino acids 114 to 120 and early truncation, thereby deleting the last two-thirds of the enzyme protein.

The deletion/insertion mutation, g7902ACT→G, was, to our knowledge, the first insertion/deletion mutation reported in the

HMB-synthase gene (Human Gene Mutation Database). This mutation results in a frameshift with 10 different residues substituted into the polypeptide prior to truncation of 73 C-terminal amino acids. The mechanism responsible for this complex mutation was not clear, as there were no direct repeats or sequences that might have predisposed this region to slipped mispairing or other mutational events.

The six other unrelated Spanish AIP probands had five mutations (G111R, R116W, R149X, R167W, and R173W in two unrelated families) previously identified in other ethnic groups (for review, see ref. 18). Of note, four of the five previously reported mutations (R116W, R149X, R167W, and R173W) occurred at CpG dinucleotides, known hotspots of mutation (19) consistent with multiple de novo origins. The G111R mutation was first reported in AIP patients from France and Belgium (5,20). More recently, the G111R mutation was identified in 12 of 26 presumably unrelated Argentinean AIP patients (11). Although the ethnic background of the Argentinean probands with the G111R mutation could not be determined with certainty, haplotype analysis indicated that these patients had a common ancestor with AIP. Since Argentina was originally colonized by the Spanish, it is possible that the G111R mutation arose within the Spanish population. Molecular diagnosis of additional Spanish AIP patients should reveal if G111R is a common mutation in Spain. R116W was found in 15 of 49 Dutch AIP families (12). The cause of the high frequency of R116W in the Dutch population was unclear, as there was no common ancestor among the Dutch AIP patients for at least four generations, nor were the patients haplotyped (12). Mutation R149X was first reported in a Finnish patient (21), R167W in an English homozygous AIP patient and in a Dutch patient (22,23), while R173W was initially identified in a Scottish AIP patient (24). Subsequent to these reports, each of these mutations have been detected in AIP patients from other ethnic groups (5,25).

Purified HMB-synthase from *E. coli* has been crystallized, and the structure has been determined to 1.9Å resolution (26). The crystal structure revealed three domains, each comprising β-sheets, an α-helical secondary structure, and a discrete hydrophobic core. Since the *E. coli* and human HMB-synthase amino acid sequences have about 35% amino acid identity and more than 70% similarity, it is possible to infer structure–function relationships for certain human

HMB-synthase mutations (27). Four of the previously reported mutations (R116W, R149X, R167W, and R173W) involve the replacement of invariant arginines that are important for the enzyme's structure and function (27). R116 is involved in an interdomain salt bridge and its replacement by a tryptophan renders the enzyme less stable, while mutation R149X would truncate the terminal 210 amino acids, including about 15 residues in domain 1, about 63 residues in domain 2, and all 132 residues in domain 3 (27). Mutations R167W and R173W replace arginines involved in critical salt bridges that stabilize interactions with the acetate and propionate side groups of the dipyrromethane cofactor in the active site. R167 interacts with a ring C2 side chain when the cofactor pyrrole ring occupies the putative substrate binding site and R173 similarly interacts with the side chains of rings C1 and C2 of the cofactor (27). The insertion/deletion mutation may affect the attachment of the dipyrromethane cofactor to domain 3 of the HMB-synthase protein (28). Mutation M212V is located in a loop region connecting the  $\beta 4$  and  $\beta 5$  sheets of domain 1 and may result in loss of its mobility, which is required for the entry of successive substrate molecules (26).

In summary, the first HMB-synthase mutations causing AIP in nine unrelated Spanish probands were identified, including three novel and five previously reported mutations, four of the later involving known mutational hotspots in the gene. The finding of eight mutations in nine families highlights the molecular heterogeneity underlying AIP, enables the precise diagnosis of asymptomatic heterozygotes in these Spanish families, and provides additional information for future structure-function studies of the human enzyme.

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