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Hydroxymethylbilane Synthase Gene Mutations and Polymorphisms in Brazilian Families with Acute Intermittent Porphyria

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Summary

Acute intermittent porphyria (AIP), an autosomal dominant disorder, is caused by a deficiency of hydroxymethylbilane synthase (HMBS). In the present study, we sought to establish a correlation between HMBS activity with the presence of mutations and polymorphisms. Enzyme activity was measured in red blood cells of four Brazilian unrelated AIP families ($n = 124$) and in blood donors ($n = 80$). The *HMBS* mutations in AIP family members were studied by PCR-SSCP followed by direct sequencing. Six intragenic SNPs (1345 G>A, 1500 T>C, 2377 C>A, 2478 A>G, 3581 A>G, and 7064 C>A) were determined by PCR-RFLP. Abnormal SSCP patterns in exons 7, 9, 12, and 15 were observed. DNA sequencing analysis revealed one nonsense mutation, R149X, two missense mutations, G111R and L338P, and one deletion, CT 730–731. All mutation carriers had lower enzyme activity. All polymorphisms, except 2377 C>A and 7064 C>A, showed no significant differences compared with previous reports. Mutation screening allowed the detection of the missense mutation, L338P, and the 730_731delCT deletion, two as yet unreported mutations in Brazilian AIP patients. Our findings also showed a high frequency of 2478 A>G and 3581 A>G polymorphism combinations suggesting that these polymorphisms contributed to enzymatic activity reduction in our study population.

Keywords: Acute intermittent porphyria (AIP), hydroxymethylbilane synthase (HMBS), enzyme activity, single-stranded conformational polymorphism (SSCP), DNA sequencing, restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR), single nucleotide polymorphism (SNP)

Introduction

Acute intermittent porphyria (AIP; OMIM 176000) is an inherited autosomal dominant disease caused by an activity deficiency in hydroxymethylbilane synthase (HMBS) the third enzyme of the heme biosynthesis pathway. AIP is a metabolic disease which results in attacks of abdominal pain, constipation, vomiting, and neurological signs and symptoms (Kappas et al., 1995), all of which are probably related to increases in heme metabolites at the onset of the crisis (Medenica et al., 1997). However, only 10% to 20% of the carriers present clinical manifestations (Kauppinen & Mustajoki, 1992). The disease prevalence is higher in women, who may present an acute crisis usually at the premenstrual period suggesting that

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the female hormones may play a role in liver heme synthesis (Granick, 1966; McColl et al., 1982; De Block et al., 1999). As there is a 50% chance of a sibling being affected, it is worthwhile detecting the carriers so that preventive measures may be taken (Kappas et al., 1995). Usually the diagnosis is based upon levels of urine porphyrins and their precursors during the crisis. Red cell HMBS activity assaying is performed by spectrofluorimetry or by spectrophotometry (Puy et al., 1997; Grandchamp et al., 1976). However, levels of enzyme activity are insufficient to confirm that a decreased activity may identify a carrier, as enzyme levels may overlap between normal controls and carriers (Bottomley et al., 1981; Bonaitie-Pellié et al., 1984). Therefore, mutation and polymorphism detection has been successfully employed to identify AIP carriers.

The *HMBS* gene has been cloned and sequenced (Raich et al., 1986; Grandchamp et al., 1987), and is 10 Kb in length with 15 exons which are transcribed by two distinct promoters. The housekeeping promoter is in the 5′ flanking region and is expressed in all tissues while the other promoter is in intron 1 and is active only in erythroid tissue (Yoo et al., 1993). Since the first *HMBS* mutation was described (Grandchamp et al., 1989), more than 380 different mutations (available at http://www.hgmd.cf.ac.uk/ac/gene.php?gene=HMBS) and 21 polymorphisms have been reported, revealing a great variability for this metabolic disease (Hrdinka et al., 2006; Brancaleoni et al., 2012). This finding suggests that *HMBS* is very prone to mutations which are usually family specific, since in only a few cases has the same mutation been found in nonrelated AIP patients. For example, some populations may present the same mutation, as with the missense mutation R173W which occurs in nonrelated Scottish patients (Greene-Davis et al., 1997) and G111R detected in 12 Argentine families (De Siervi et al., 1999). This molecular variability requires gene investigation of the 15 exons by single-stranded conformational polymorphism (Kauppinen et al., 1995), denaturing gradient gel electrophoresis (Gu et al., 1994; Puy et al., 1997), chemical cleavage mismatch (Ong et al., 1998), heteroduplex (Schreiber et al., 1995) or high-resolution melting analysis (Ulbrichova-Douderova & Martasek, 2009).

Before molecular analysis of the HMBS gene became available, the diagnosis of mutation carriers usually relied upon the enzymatic activity measurement. However, the enzyme assay has a sensitivity of approximately 90% and, owing to the overlap between high pathologic and low normal values, its diagnostic reliability is limited (Bonaitie-Pellié et al., 1984). The detection of intragenic polymorphisms of the *HMBS* gene by restriction fragment length polymorphisms (RFLPs) enables tracking of the AIP gene in affected families and allows asymptomatic carriers and normal individuals to be identified with greater certainty than can be achieved by enzymatic methods alone (Llewellyn et al., 1987; Lee et al., 1988; Lee et al., 1990). However, this approach is confined to the families of patients who have both potentially informative genotypes and sufficient unequivocally affected, living relatives to enable linkage to be established (Scobie et al., 1990).

The aim of this study was to establish a correlation between HMBS activity with the presence of mutations and polymorphism in the Brazilian population using four AIP families and 80 unrelated blood donors. Mutation screening was evaluated by PCR-SSCP followed by direct sequencing and six known intragenic SNPs in the human HMBS gene, namely: 1345 G>A (rs1799991), 1500 T>C (rs1799992), 2377 C>A (rs 1799993), and 2478 A>G (rs1799994) in intron 1, 3581 A>G (rs17075) in intron 3, and 7064 C>A (rs1784304) in intron 10 were analyzed by PCR-RFLP.

Material and Methods

AIP Subjects and Control Group

Four patients of the neurology sector from the University of São Paulo Faculty of Medicine Clinics Hospital (HC-FMUSP) were included in this study. They presented previously with AIP crisis and were diagnosed by increased excretion of δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) in the urine. Family members ($n = 124$; 67 men and 57 women) of the probands were enrolled from different Brazilian regions; Family D ($n = 60$) from São Paulo City, family S ($n = 26$) from Ilicinia – Minas Gerais, family SP ($n = 21$) from Aracaju – Sergipe, and family B ($n = 17$) from Bebedouro – São Paulo. Seventy individuals from São Paulo city, without AIP history and unrelated to the AIP families, were used to measure HMBS activity and mean ± standard deviation was used to create reference values. This study was approved by the Ethics Committee of the University of São Paulo Faculty of Medicine Clinics Hospital (number: 001/99) and all individuals recruited gave informed written consent.

Blood Donors

The blood donor group was composed of 80 (58 men and 22 women) voluntary blood donors from Antonio Pedro University Hospital, Niterói, RJ, with no reports of AIP in the families. Informed written consent was obtained from all participants, and the study was approved by the local Ethics Committee (number 090/07).

HMBS Activity

Erythrocyte HMBS activity was evaluated by spectrofluorimetric (Puy et al., 1997) or spectrophotometric assays (Grandchamp et al., 1976) using 5 mL of blood. Reference

range values were calculated using mean ± standard deviation of the measured HMSB activity. The AIP families D, S, SP, and B, were analyzed by spectrofluorimetry and a reference range was based on the control samples ($n = 70$) for whom enzyme activity ranged from 136.1 to 198.5 (mean±SD) pmol uro/mg Hb at 37 °C/h. As the spectrofluorimetric methodology was not available in Niterói city, the HMBS activity of the blood donor group was analyzed by spectrophotometry obtaining a reference range from 129.7 to 216.7 (mean ± SD) pmol uro/mg Hb at 37 °C/h. The *AIP* family members and blood donors with HMBS activity below these reference values were considered deficient. Enzyme activity results of AIP mutated carriers were considered as overlapping values when overlapped with low normal enzyme activity values.

DNA Extraction

Blood collected in ethylenediaminetetraacetic acid (EDTA; 5mL) was used in DNA extraction as described by Lahiri & Nurnberger (1991) and adapted by Salazar et al. (1998).

PCR-Single-Strand Conformation Polymorphism Analysis

All 15 HMBS exons were amplified using the same primers and protocols described by Schreiber et al. (1994), which amplify all exons and at least 10 to 20 bases of flanking intronic sequences, with minor modifications described by Ribeiro et al. (2002).

Automated Sequencing

The DNA fragments showing abnormal SSCP patterns were purified with a Gel Band Purifying Kit (Amersham Life Sciences, Cleveland, Ohio, USA) and directly sequenced with a BigDye™ Terminator Cycle Sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were analyzed on an ABI Prism 377 Sequencing System (Applied Biosystems). Detected mutations were confirmed in the sense and antisense strand and were absent in the controls.

Family Study

Based on the proband's SSCP abnormal pattern, all family relatives were evaluated in order to detect possible presymptomatic carriers.

Polymorphisms Analysis

Six known intragenic SNPs in the human *HMBS* gene, namely: 1345 G>A, 1500 T>C, 2377 C>A, 2478 A>G in intron 1, 3581 A>G in intron 3, and 7064 C>A in intron 10, were analyzed in all 124 individuals from the four AIP families. In addition, the six SNPs were analyzed in 80 blood donors. All polymorphisms were determined by restriction enzyme digestion (*Msp* I, *Pst* I, *Apa* LI, *Bst* NI, *Bsm* AI and *Hinf* I, purchased from New England BioLabs Inc. Ipswich, MA, USA) of the PCR products and followed by fragment-length analyses in a 2% agarose gel, as described by Yoo et al. (1993) and Schreiber et al. (1994).

Statistical Analysis

The polymorphism allele distributions were tested for Hardy-Weinberg equilibrium. Allelic and genotype frequencies were calculated by direct counting and statistical comparisons between groups were performed by the Fisher's exact test; odds ratios (OR) with 95% confidence intervals (95%CI) was calculated. Statistical analysis was performed using GraphPad InStat software (3.0 version, Inc., San Diego, CA, USA) and $P < 0.05$ values were considered to be statistically significant.

Results

HMBS Activity

Based on enzymatic activity, reference values were observed which indicated that 43.3% (26/60) of family D individuals were deficient, while these indices were 53.9% (14/26) in S family, 28.6% (6/21) in SP family and 70.6% (12/17) in B family.

Mutation Analysis and Family Screening

Agarose gel electrophoresis of the amplified DNA fragments for exons 1–15 and the promoter region of the *HMBS* gene showed single bands ranging from 140 to 375 base pairs. The four symptomatic AIP patients, one from each family, were analyzed by PCR-SSCP and were compared to controls. Four altered patterns of mobility for exons 7, 9, 12, and 15 were observed. The results of family D SSCP analysis showed an extra band at exon 7 and sequencing analyses revealed a missense mutation G111R (Ribeiro et al., 2002); 23 out of 60 (38.3%) individuals were mutated in this family.

In family S, an extra band was observed in exon 9 of the proband. After direct sequencing, the nonsense mutation R149X, a C to T transition at position 445 which leads to an arginine to stop codon change (Ribeiro et al., 2002),

was identified in nine out of 26 (34.6%) individuals from this family. This mutation was found in three symptomatic relatives and also in five more asymptomatic carriers, two of whom had reported sporadic and mild symptoms which could be related to AIP.

After screening all *HMBS* exons of family SP, a different pattern was found in the fragment containing exon 12. Direct sequencing revealed the deletion delCT$^{730–731}$ which caused a frameshift resulting in premature termination at residue 250. Of all 21 available relatives analyzed, four mutated individuals were observed (19.0%).

The results of SSCP analysis in family B showed a heteroduplex at exon 15 (Fig. 1A). Sequence analysis disclosed a missense mutation, L338P, due to a transition of T to C at position 1040 (Fig. 1B) in seven individuals. The heredogram of family B (Fig. 1C) illustrates the results obtained. It was possible to identify the proband and six asymptomatic carriers (41.2%) in 17 relatives available in this family.

The mean activity values of AIP mutated carriers was 29.2% to 41% lower than nonmutated family members ($P < 0.001$). There was no difference when the nonmutated and control groups were compared ($P > 0.05$).

Polymorphism Analysis and HMBS Activity

The allelic and genotypic frequencies of the six polymorphisms observed in the blood donors and AIP families are described in Table 1. All the polymorphism distributions in the analyzed groups were in Hardy-Weinberg equilibrium ($P > 0.05$). When comparing families with AIP history with the blood donor group ($n = 80$), different allelic and/or genotypic distributions were found for polymorphisms 1345 G>A, 1500 T>C, 2377 C>A, 2478 A>G, 3581 A>G, and 7064 C>A in family D; 1345 G>A, 2377 C>A, 3581 A>G, and 7064 C>A in family S; 1345 G>A and 2478 A>G in family SP, and 1345 G>A, 3581 A>G, and 7064 C>A, in family B. Based on these results, we analyzed the polymorphisms' distribution in the presence or absence of the disease-causing mutations in family members. No significant difference ($P > 0.05$) was observed when analyzing the distribution of polymorphisms in the aforementioned groups of family members with a history of AIP who inherited the mutations G111R, R149X, and 730–731delCT (families D, S, and SP) with those family members not carrying the mutations. However, polymorphism 7064 C>A in family B had a different distribution ($P = 0.0408$) among mutation carrier and noncarrier family members, with the allele A frequency 86% and 45%, respectively (OR = 7.0; CI = 1.291–41.670). The 7064 C>A polymorphism in Family B was probably related to the L338P mutation, the cause of the disease in this family.

In order to identify if any of the polymorphisms segregated with the enzymatic deficiency phenotype, the 80 blood donors were designated nondeficient and deficient based on HMBS activity; 14 (17.5%) were classified as deficient (Table 2). Using the nondeficient group as a reference and comparing with mutated AIP family members, all polymorphisms had a different distribution except the 7064 C>A polymorphism ($P > 0.05$). Deficient nonmutated AIP family members ($n = 15$), who presented enzymatic activity deficiency and absence of mutation, were also compared with the reference group. The 2478 A>G and 3581 A>G polymorphisms had different distributions ($P < 0.05$).

In comparing nondeficient with deficient blood donors, it was observed that only the 1345 G>A polymorphism had a difference in distribution and the A allele was associated with deficiency ($P = 0.02$). In contrast, in the AIP mutated family members the G allele was the risk allele. We also observed a concomitant higher frequency of genotypes containing the polymorphic alleles of 2478 A>G and 3581 A>G polymorphisms in enzymatic deficient individuals (Table 2).

Discussion

AIP is a disease which exhibits great clinical variability and the detection of carriers is difficult when based only on red cell HMBS activity or urine ALA and PBG assays. As PCR-SSCP screening has been widely used to safely detect the mutations (Kauppinen et al., 1995), it was used in this study and included the exons, exon-intron junctions, and the promoter region. This approach has been recommended as the genetic alterations may occur throughout the entire gene and are usually family specific (Kauppinen et al., 1995; Puy et al., 1997).

Previous results from our group using PCR-SSCP and direct sequencing allowed the detection of the nonsense mutations, R149X, R225X, and R325X, and the missense mutation, G111R, in Brazilian AIP patients. In addition, intragenic polymorphisms, 65 C>T, 3119 A>G, 3581 G>A, 7052 G>A, and 7064 C>A, and two silent mutations, V202V and A266A, were detected (Ribeiro et al., 2002). Three nucleotide deletion (IVS3+2_4delTGG) were also found in a patient with overt AIP disease (Ribeiro et al., 2007).

In this study, four probands and their 120 family members were included and family- specific mutations were found. The G111R mutation, found in family D from São Paulo, was originally described in Europe (Gu et al., 1993). It was also reported in Slavic (Rosipal et al., 1997), French (Puy et al., 1997), British (Whatley et al., 1999), Swedish (Andersson et al., 1995; and Floderus et al., 2002) and Polish patients (Gregor et al., 2002). In Argentina, the G111R mutation was detected in 12 families (De Siervi et al., 1999) which seem

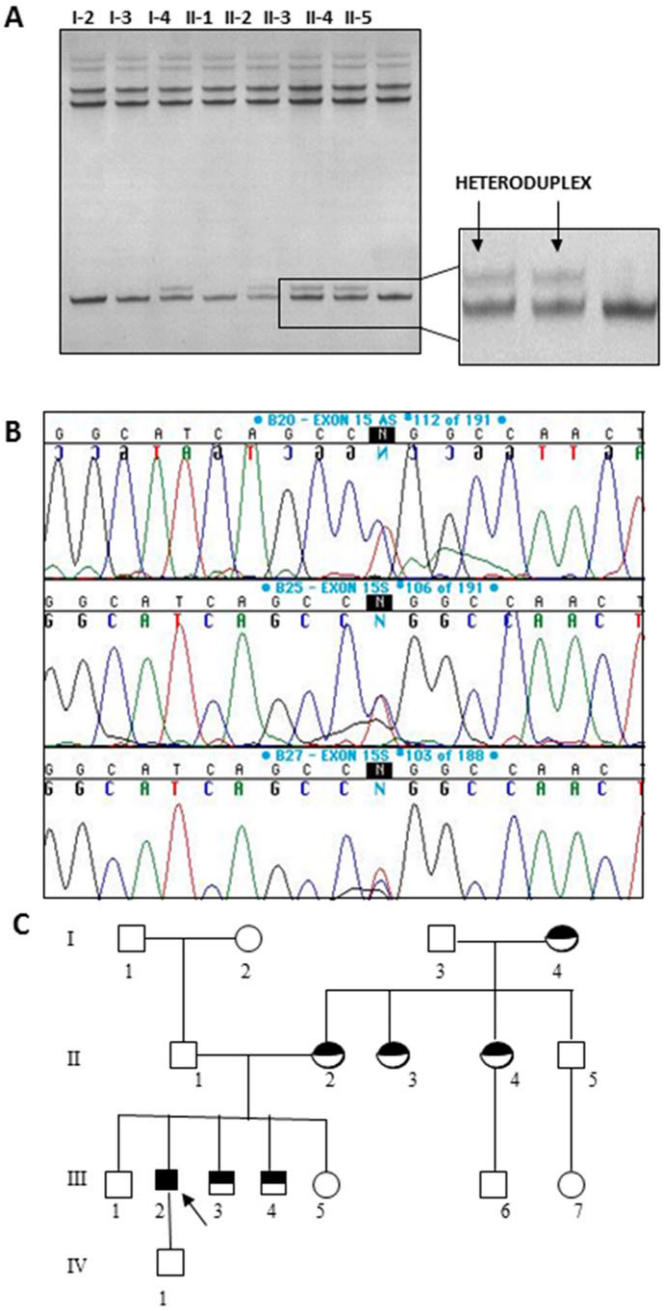

Figure 1 Analysis of the AIP family B *HMBS* mutation by PCR-SSCP and direct sequencing.
Exon 15 of the *HMBS* gene was analyzed by PCR-SSCP (A). Normal individuals showed one band whereas a heteroduplex was observed in asymptomatic gene carriers. (B). The mutation L338P indicated by the altered pattern was characterized by direct sequencing (C). The heredogram illustrates the mutation carriers and proband individual completely blackened symbol denotes patient with overt AIP and half blackened symbols denote asymptomatic carriers. Family screening was based on DNA extraction followed by one PCR and one SSCP of the affected exon 15.

Table 1 Frequency of *HMBS* gene polymorphisms in blood donors and AIP families from Brazil.

	Blood donors		AIP families							
	($n = 80$)		D ($n = 60$)		S ($n = 26$)		B ($n = 17$)		SP ($n = 21$)	
Polymorphism	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1345 G>A (*Msp* I)										
GG	22	27.5	30	50.0	0	0.0	1	5.9	1	4.8
AG	44	55.0	29	48.3	23	88.5	12	70.6	13	61.9
AA	14	17.5	1	1.7	3	11.5	4	23.5	7	33.3
A	*0.550*		*0.742*		*0.442*		*0.412*		*0.357*	
G	*0.450*		*0.258*		*0.942*		*0.588*		*0.643*	
1500 T>C (*Pst* I)										
TT	36	45.0	38	63.3	12	46.2	10	58.8	12	57.1
TC	35	43.8	21	35.0	14	53.8	7	41.2	6	28.6
CC	9	11.3	1	1.7	0	0.0	0	0.0	3	14.3
T	*0.669*		*0.808*		*0.731*		*0.794*		*0.714*	
C	*0.331*		*0.192*		*0.538*		*0.206*		*0.286*	
2377 C>A(*Apa* LI)										
AA	18	22.5	1	1.7	0	0.0	2	11.8	4	19.0
CA	43	53.8	36	60.0	22	84.6	6	35.3	11	52.4
CC	19	23.8	23	38.3	4	15.4	9	52.9	6	28.6
A	*0.494*		*0.317*		*0.423*		*0.294*		*0.452*	
C	*0.506*		*0.683*		*0.923*		*0.706*		*0.548*	
2478 A>G (*Bst* NI)										
AA	41	51.3	3	5.0	9	34.6	4	23.5	4	19.0
AG	29	36.3	27	45.0	14	53.8	12	70.6	12	57.1
GG	10	12.5	30	50.0	3	11.5	1	5.9	5	23.8
A	*0.694*		*0.275*		*0.615*		*0.588*		*0.476*	
G	*0.306*		*0.725*		*0.596*		*0.412*		*0.524*	
3581A>G (*Bsm*AI)										
AA	34	42.5	4	6.7	9	34.6	2	11.8	4	19.0
AG	36	45.0	35	58.3	14	53.8	6	35.3	12	57.1
GG	10	12.5	21	35.0	3	11.5	9	52.9	5	23.8
A	*0.650*		*0.358*		*0.615*		*0.294*		*0.476*	
G	*0.350*		*0.642*		*0.596*		*0.706*		*0.524*	
7064 C>A (*Hinf* I)										
AA	7	8.8	3	5.0	16	61.5	6	35.3	3	14.3
CA	31	38.8	13	21.7	10	38.5	9	52.9	9	42.9
CC	42	52.5	45	75.0	0	0.0	2	11.8	9	42.9
A	*0.281*		*0.158*		*0.808*		*0.618*		*0.357*	
C	*0.719*		*0.858*		*0.385*		*0.382*		*0.643*	

Italics mean allele frequency

to have been due to a founder effect. In family S, the disease was caused by the R149X mutation that was first identified in Finland by Kauppinen et al. (1995). This mutation probably accounts for the AIP in this family and is also related to patients diagnosed with porphyria in Poland (Gregor et al., 2002) and France (Puy et al., 1997). A missense mutation, L338P, identified in family B residing in Bebedouro city, São Paulo, was only described by Surin et al. (2010) in a study conducted in Russia. The proband's parents were of Iberic extraction, very distant and with no reported slavish migration to Iberic countries; this suggests the hypothesis of a de *novo mutation* affecting the Brazilian patient.

The deletion 730_731delCT described in a Danish patient (Mgone et al., 1993) was found in family SP, resident in Sergipe, but previously it has also been reported independently worldwide, including in Finland (Kaupinen et al.,

Table 2 *HMBS* gene polymorphism frequency and HMBS enzyme activity.

	Blood donors				AIP families with mutation ($n = 43$)		Deficient AIP families without mutation ($n = 15$)		AIP families with mutation versus nondeficient donor		Deficient AIP families without mutation versus nondeficient donor		Deficient blood donor versus nondeficient donor	
	Deficient ($n = 14$)		Non-deficient ($n = 66$)											
Polymorphism	*n*	%	*n*	%	*n*	%	*n*	%	OR(IC)	*P*	OR(IC)	*P*	OR(IC)	*P*
1345 G>A (*Msp* I)														
GG	7	50.0	15	22.7	2	4.7	0	0.0						
AG	7	50.0	37	56.1	25	58.1	11	73.3	0.197(0.041–0.939)	**0.0425**	0.105(0.006–1.899)	0.053	2.467(0.737–8.252)	0.2416
AA	0	0.0	14	21.2	16	37.2	4	26.7	0.117(0.023–0.602)	**0.0057**	0.104(0.005–2.107)	0.108	14.032(0.732–268.54)	0.0549
AG+AA	7	50.0	51	77.3	41	95.3	15	100.0	0.166(0.036–0.767)	**0.0231**	0.107(0.006–1.897)	0.061	3.400(1.028–11.240)	0.0808
A	*0.750*		*0.508*		*0.337*		*0.367*							
G	*0.250*		*0.492*		*0.663*		*0.633*		*0.494(0.281–0.866)*	***0.0175***	*0.562(0.248–1.272)*	*0.224*	*2.910(1.159–7.312)*	***0.0219***
1500 T>C (*Pst* I)														
TT	9	64.3	27	40.9	29	67.4	9	60.0						
TC	5	35.7	30	45.5	14	32.6	6	40.0	2.302(1.011–5.242)	0.0666	1.667(0.524–5.299)	0.563	2.000(0.596–6.713)	0.3723
CC	0	0.0	9	13.6	0	0.0	0	0.0	20.382(1.131–367.32)	**0.0033**	6.564(0.348–123.99)	0.169	6.564(0.347–123.99)	0.1689
TC+CC	5	35.7	39	59.1	14	32.6	6	40.0	2.992(1.338–6.692)	**0.0120**	2.167(0.690–6.801)	0.251	2.600(0.789–8.620)	0.1932
T	*0.821*		*0.636*		*0.837*		*0.800*							
C	*0.179*		*0.364*		*0.163*		*0.200*		*2.939(1.498–5.764)*	***0.0013***	*2.286(0.873–5.985)*	*0.132*	*2.629(0.938–7.365)*	*0.0766*
2377 C>A (*Apa* LI)														
CC	4	28.6	15	22.7	17	39.5	5	33.3						
CA	10	71.4	33	50.0	26	60.5	9	60.0	1.438(0.606–3.413)	0.5104	1.222(0.349–4.276)	0.755	0.880(0.237–3.269)	1.000
AA	0	0.0	18	27.3	0	0.0	1	6.7	41.774(2.318–752.95)	**<0.0001**	6.000(0.630–57.17)	0.182	10.742(0.535–215.63)	0.1050
CA+AA	10	71.4	51	77.3	26	60.5	10	66.7	2.223(0.959–5.149)	0.0953	1.700(0.503–5.749)	0.507	1.360(0.372–4.965)	0.9037
C	*0.643*		*0.477*		*0.698*		*0.633*							
A	*0.357*		*0.523*		*0.302*		*0.367*		*2.527(1.425–4.484)*	***0.0014***	*1.471(0.655–3.305)*	*0.423*	*1.971(0.847–4.591)*	*0.1454*
A2478G (*Bst* NI)														
AA	4	28.6	37	56.1	7	16.3	2	13.3						
AG	7	50.0	22	33.3	20	46.5	11	73.3	0.208(0.075–0.571)	**0.0023**	0.108(0.022–0.534)	**0.004**	0.340(0.089–129)	0.1806
GG	3	21.4	7	10.6	16	37.2	2	13.3	0.083(0.025–0.275)	**<0.0001**	0.189(0.023–1.577)	0.155	0.254(0.046–1.383)	0.1260
AG+GG	10	71.4	29	43.9	36	83.7	13	86.7	0.152(0.059–0.392)	**<0.0001**	0.121(0.025–0.578)	**0.004**	0.313(0.891–1.103)	0.1153
A	*0.536*		*0.727*		*0.395*		*0.500*							
G	*0.464*		*0.273*		*0.605*		*0.500*		*0.245(0.138–0.437)*	*<0.0001*	*0.444(0.196–1.006)*	*0,056*	*0.433(0.188–0.998)*	*0.0691*
3581 A>G (*Bsm* AI)														
AA	4	28.6	30	45.5	7	16.3	1	6.7						
AG	6	42.9	30	45.5	18	41.9	11	73.3	0.389(0.142–1.067)	0.0923	0.091(0.011–0.749)	**0.010**	0.667(0.171–2.605)	0.7356
GG	4	28.6	6	9.1	18	41.9	3	20.0	0.078(0.023–0.268)	**<0.0001**	0.067(0.006–0.755)	**0.030**	0.200(0.0388–1.031)	0.0641
AG+GG	10	71.4	36	54.5	36	83.7	14	93.3	0.233(0.090–0.599)	**0.0033**	0.086(0.011–0.690)	0.007	0.480(0.136–1.687)	0.3881
A	*0.500*		*0.682*		*0.372*		*0.433*							
G	*0.500*		*0.318*		*0.628*		*0.567*		*0.311(0.175–0.555)*	***<0.0001***	*0.357(0.159–0.802)*	***0.020***	*0.467(0.204–1.067)*	*0.0820*
7064 C>A (*Hinf* I)														
CC	5	35.7	37	56.1	25	58.1	8	53.3						
CA	6	42.9	25	371 .9	13	30.2	7	46.7	1.299(0.560-3.012)	0.6718	0.7722(0.248–2.01)	0.772	0.563(0.155–2.048)	0.5110
AA	3	21.4	4	6.1	5	11.6	0	0.0	0.540(0.132–2.213)	0.4791	2.04(0.100–41.63)	1.000	0.180(0.031–1.052)	0.0753
CA+AA	9	64.3	29	43.9	18	41.9	7	46.7	1.089(0.500–2.368)	0.9870	0.278(0.080–0.970)	0.050	0.435(0.132–1.441)	0.2757
C	*0.571*		*0.750*		*0.733*		*0.767*							
A	*0.429*		*0.250*		*0.267*		*0.233*		*0.913(0.492–1.696)*	*0.8741*	*1.095(0.433–2.786)*	*1.000*	*0.444(2.191–1.036)*	*0.0664*

Bold numbers; $P < 0.05$ and Italics mean allele frequency.

1995), Holland, and France (Puy et al., 1997), Argentina (De Siervi et al., 1999), Italy (Martinez di Montemuros et al., 2001), Sweden (Floderus et al., 2002), Poland (Gregor et al., 2002), Israel (Ulbrichova et al., 2009), and Japan (Susa et al., 2013). Mutation detection in AIP families allows the identification of latent porphyria carrier and non-carrier members who may be confidently discriminated. The AIP noncarrier assignment avoids restrictions to prevent AIP attacks and eliminates the need for offspring screening.

The molecular results from the SSCP analysis indicate that a mutation related to the deficient enzyme was associated with the AIP condition, although in family D three deficient individuals (5%) did not carry the mutation, while in families S, B, and SP this index was 5 (19.2%), 5 (29.4%), and 2 (9.5%) respectively.

In some cases, levels of enzyme activity can result in false-positives for AIP, because there is an overlapping zone between individuals with the disease and healthy individuals with low normal enzyme activity, probably as a consequence of the interaction of various endogenous and exogenous factors, making a final diagnosis difficult (Magnussen et al., 1974; Bonaiti-Pellié et al., 1984; Bonkovsky & Barnard, 1998). Furthermore, Peterson et al. (1976) reported considerable variation in enzyme activity, even among individuals who expressed porphyria and as well as in possible latent cases, so that only the molecular diagnosis correctly shows all patients with AIP (Kauppinen et al., 1995).

This present study enrolled 204 individuals, of whom 80 were volunteer blood donors of the HUAP blood bank and 124 were members of families with an AIP history. These families had one or more members who previously expressed moderate or serious disease. Based on HMBS activity, 14 blood donors were classified as deficient individuals and 58 individuals of AIP families were classified as deficient. Subsequently, when performing molecular investigation by PCR-SSCP and sequencing, it was possible to confirm which individuals actually suffered from AIP and thus confirm those family members who had been misclassified. We observed that mutated individuals had lower enzyme activity when compared with nonmutated subjects ($P < 0.001$) and identified 15 deficient individuals in AIP families who were not mutation carriers. To verify whether polymorphisms in the *HMBS* gene influenced the reduction of enzyme activity, thus contributing to the overlapping zone, the distribution of six polymorphisms was evaluated.

Comparing Brazilian blood donors analyzed in this study (Table 1) with the worldwide distribution of polymorphism frequency reported by Hrdinka et al. (2006), we observed that 2377 C>A and 7064 C>A showed no significant difference. The 2478 A>G polymorphism had a different distribution from the Finnish and European populations described by Scobie et al. (1990). The 3581 A>G polymorphism also had a different distribution when compared with those of Japan and Africa (Daimon et al., 1993; Robreau-Fraolini et al., 2000). The 1345 G>A polymorphism was different only when compared with the Finnish population (Kauppinen et al., 1990).

Before the development of molecular methods for mutation screening in the *HMBS* gene, polymorphisms were used as markers to identify asymptomatic heterozygotes (Yoo et al., 1993; Law et al., 1999). In a study by Lee et al. (1988), analyzing the results of RFLP for 1345 G>A, 2478 A>G, and 1500 T>C polymorphisms, it was important to discriminate between two individuals who were considered potential carriers of the disease, because the amounts of enzyme activity, in both cases, were in the overlapping zone. From the analysis of the allele frequency of polymorphisms related to haplotypes, it was proven that neither individual had inherited the mutant allele from their relatives with AIP. These analyses establish the carrier status of gene mutation in families with the disease. In order to assess whether any polymorphism is related to the deficiency enzyme phenotype, the polymorphism frequency in nondeficient blood donors was compared with that in mutated AIP family members, deficient nonmutated AIP family members and deficient blood donors.

Among the three deficient groups analyzed, it was observed that 2478 A>G, and 3581 A>G polymorphisms, located in intron 1 and 3, respectively, were significantly associated with enzyme deficiency, suggesting that these polymorphisms were contributing to the low enzyme activity. Yoo et al. (1993), describing the complete sequence of the human *HMBS* gene, observed that these polymorphisms were in linkage disequilibrium and should facilitate prediction of AIP carriers in which the specific mutations have not been identified. In that study, although putative regulatory motifs regions for the erythroid-specific promoter had been located in intron 1, the 2478 A>G polymorphism was not in this region. We also observed that the polymorphic alleles of 2478 A>G and 3581 A>G polymorphisms occur in the same individual of deficient blood donors, deficient nonmutated and mutated AIP family members, suggesting that these polymorphism combinations contributed to the reduction in enzymatic activity in our population. Further studies exploring the enzymatic deficiency phenotype and the *HMBS* gene will be required to explain this association.

Conclusion

Genomic DNA screening by PCR-SSCP and direct sequencing allowed the detection of the missense L338P and 730_731delCT two previously unreported mutations in Brazilian AIP patients. It was also possible to confirm 43 AIP carriers and exclude 15 individuals who had levels of enzyme activity in the overlapping zone. This strategy allows latent

AIP carrier detection, which may prevent the onset of clinical acute manifestations of the disease, by recommending avoidance of the known triggering agents. If molecular diagnosis is possible, it must be done for the screening of latent carriers because ALA and PBG are usually estimated during the symptomatic phase of AIP. Among the six polymorphisms studied only polymorphisms 2377 C>A and 7064 C>A showed no significant difference in frequency distribution when compared with reported data. The 7064 C>A polymorphism in family B was probably related to the L338P causal mutation in this family. Considering the enzyme phenotype, we observed a high frequency of 2478 A>G and 3581 A>G polymorphism combinations in individuals deficient for HMBS activity, suggesting that these polymorphisms contributed to enzymatic activity reduction. However, this association needs to be confirmed in a study using a larger and independent cohort.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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