Porphobilinogen Deaminase Gene Mutations in Brazilian Acute Intermittent Porphyria Patients

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Acute intermittent porphyria (AIP) is an autosomal dominant disorder resulting from porphobilinogen deaminase (PBGD) deficiency. Seven unrelated Brazilian patients were investigated regarding PBGD gene mutations by polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) analysis followed by direct DNA sequencing. The PBG gene screening disclosed abnormal SSCP patterns in exons 7, 9, 12, 13, and 15, as well as in introns 3 and 10. Direct DNA sequencing revealed the occurrence of three nonsense mutations (R149X, R225X, and R325X) in exons 9, 12, and 15, respec-

tively, and one missense mutation G111R in exon 7. The G111R mutation was detected in two unrelated patients. Intragenic polymorphisms (3119 G/T in intron 2, 3581 G/A in intron 3, 7052 A/G and 7064 C/A in intron 10, and –65 C/T in exon 1) were also observed. In addition, two silent mutations (V202V in exon 10 and A266A in exon 13) were found. The latter has not heretofore been reported. Thus, this study revealed the mutations involved in Brazilian symptomatic AIP patients, as well as the intragenic polymorphisms found in the patients. *J. Clin. Lab. Anal.* 16:259–265, 2002. © 2002 Wiley-Liss, Inc.

Key words: PBGD gene; PCR-SSCP; acute intermittent porphyria

INTRODUCTION

Acute intermittent porphyria (AIP) is an inherited autosomal dominant disease caused by deficiency of porphobilinogen deaminase (PBGD), the third enzyme of heme biosynthesis. AIP is a metabolic disease clinically characterized by attacks of abdominal pain, constipation, and vomiting, as well as neurologic signs and symptoms (1). However, only 10–20% of the carriers present clinical manifestations (2). The disease prevalence is higher in females, who usually develop an acute crisis during the premenstrual period, suggesting that some sex hormones may play a role in the liver heme synthesis (3). As there is a 50% chance that a sibling will be affected, it is worthwhile to detect the carriers, because preventive measures should be undertaken (1).

The entire porphobilinogen deaminase (PBGD) gene has been cloned and sequenced (4,5). The PBGD is encoded by a single gene of 10 kb length located in

chromosomal region 11q24.1 → 24.2 (6); it consists of 15 exons transcribed by two distinct promoters. The housekeeping promoter is located at the 5' flanking region and its transcript is encoded by exons 1 and 3–15. The erythroid-specific promoter is located in intron 1 and generates a transcript encoded by exons 2–15 (7).

Since the PBGD mutation was first described (8), more than 100 mutations have been reported, revealing a great variability for this metabolic disease. This fact suggests that PBGD is very prone to mutations. The mutations are usually family-specific, although the same

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mutation has been described in nonrelated AIP patients. Some populations may present the same mutation, such as the nonsense mutations W198X (which occurs in nonrelated Swedish patients (9)) and W283X (found in Swiss patients (10)), and the missense mutation G111R (detected in 12 Argentine AIP families (11)). Because of this molecular variability, the investigation of PBGD gene mutations requires the examination of all 15 exons and surrounding intronic sequences. Several methods have been reported for PBGD mutation screening, such as single strand conformation polymorphism (SSCP) (12), denaturing gel gradient electrophoresis (DGGE) (13), chemical cleavage mismatch (CCM) (14), and heteroduplex (15). In the present study, PCR-SSCP and direct DNA sequencing were employed to investigate the molecular basis of AIP in seven symptomatic, unrelated Brazilian patients. The examinations revealed four disease-related mutations, two silent mutations, and five intragenic polymorphisms.

MATERIAL AND METHODS

Blood samples were obtained from seven unrelated Brazilian AIP patients (P1–P7) who had been diagnosed at the Neurology Department of the Hospital das Clínicas of Faculdade de Medicina da USP-SP and Policlínica de Especialidades CPN-Niterói, Rio de Janeiro. The diagnosis of AIP was established based on typical clinical symptoms, family history, and increased 5-aminolevulinic acid and PBG urine excretion. Peripheral blood samples from probands and 10 control subjects were also obtained. This study was approved by the institutional ethics committee, and signed informed consent was obtained from all subjects.

Methods

DNA extraction

DNA was obtained according to the method of Salazar et al. (16). First, 5 mL of blood were collected in EDTA. The white blood cells were isolated and lysed (1:10 V/V) with Tris buffer 1 (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA pH 8.0, and 25 mL/L Triton X100) and Tris buffer 2 (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA, pH 8.0, 0.4 mol/L NaCl, and 10 g/L sodium dodecyl sulfate) and incubated for 15 min at 56°C. Cellular proteins were removed by precipitation with 5 M NaCl, and DNA was isolated by ethanol precipitation and solubilized in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA).

Polymerase chain reaction (PCR) followed by SSCP

All PBGD exons were amplified using the same primers and protocols described by Schreiber et al. (15). Briefly, the PCR reactions were carried out in 50 µL volume using 1.5 mM magnesium chloride, 1 unit Taq DNA polymerase (Life Technologies, Gaithersburg, MD), 200 pmol of dNTP, 15 pmol of each primer, and 50–100 ng of genomic DNA. The thermocycler was programmed to first denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 1 min, with a Thermocycler model Genius (Techne Ltd., Cambridge, MA) or Perkin–Elmer (Oak Brook, IL) 9700 thermocycler. All exons were amplified at these conditions, except for a fragment from exons 5–6, which was annealed at 55°C.

Equivalent volumes of PCR product and loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) were mixed and incubated at 85°C for 10 min and immediately placed on ice for 2 min; 4 µL from this solution were submitted to nondenaturing 12.5% polyacrylamide gel electrophoresis using the Genephor system (Amersham Life Science, Cleveland, OH), set for 2 hr 15 min at 5°C. The fragments were detected after silver staining by an automated system (Amersham Pharmacia Biotech do Brasil, Brazil) and documented under white light using the Alfa Image System (Amersham Life Science). Control samples were always run simultaneously with patient samples.

Direct DNA sequencing

Amplified DNA fragments showing abnormal SSCP patterns were purified with a gel band purifying kit (Amersham Life Science) and directly sequenced with a BigDye™ terminator cycle sequencing ready reaction kit (PE Biosystem, Perkin–Elmer, Inc., Norwalk, CT). The sequencing reactions were analyzed using an Alfa Image ABI Prism 377 sequencing system (Applied Biosystems, Foster City, PA).

RESULTS

Mutation Analysis

The entire coding sequence of the human PBGD gene, housekeeping promotor region, and exon/intron boundaries were analyzed by PCR-SSCP and direct DNA sequencing. The mutational screening was performed using a two-step strategy: the DNA from the proband and controls were amplified by PCR for all 15 exons (PCR products ranging from 140 to 375 base pairs) and submitted to PCR-SSCP analysis. The control and unrelated AIP subjects were compared, and all PCR

Fig. 1. Mutation analysis of exon 7 of PBGD gene. **A:** PCR-SSCP analysis of exon 7 showing (lane 1) an extra band in patient P1 (asterisk). The same SSCP pattern was displayed by patient P5 (not shown); (lanes 2–4) unrelated AIP patients with no mutation in this region; and (lane 5) normal control. **B:** Direct sequencing (antisense strand) revealed a G-to-A transition at position 331 corresponding to the coding sequence in patients P1 and P5.

products with different patterns at SSCP gel were subjected to direct sequencing. This strategy revealed three nonsense and one missense mutations, two silent mutations, one novel mutation, and five polymorphisms in patients and controls.

Patients P1 and P5, who are unrelated, showed a band shift mobility in exon 7 SSCP analysis (Fig. 1a). This pattern was not found in control samples or in any other patient. The sequencing analysis revealed the presence of a G-to-A transition at position 331 of the coding sequence (Fig. 1b). This transition leads to the missense mutation G111R, which leads to arginine substitution for glycine in codon 111.

The exon 9 SSCP analysis of patient P2 showed an extra band (Fig. 2a). Direct sequencing confirmed the

Fig. 2. Mutation analysis of exon 9 of the PBGD gene. **A:** PCR-SSCP analysis of exon 9 showing (lane 1) an extra band in patient P2 (asterisk); (lanes 2–4) unrelated AIP patients with no mutation in the region; and (lane 5) normal control. **B:** Direct sequencing (antisense strand) showed a C-to-T transition at position 445 corresponding to the coding sequence.

occurrence of a C-to-T transition at position 445 of the coding sequence leading to the nonsense mutation R149X, which resulted in the change of an arginine to a stop codon (Fig. 2b). This nonsense mutation was observed only in this patient. Patient P3 showed an abnormal SSCP pattern for exon 12 (Fig. 3a) as a consequence of a C-to-T base substitution at position 673, leading to the nonsense mutation R225X (Fig. 3b). After screening of all PBGD exons in the P4 proband, an abnormal migration band was observed at exon 15 (Fig. 3c) and the nonsense mutation R325X (Fig. 3d) was identified. This mutation replaces an arginine, leading to a termination codon (CGA-to-TGA) at residue 325.

Causative AIP mutations were not found in patients P6 and P7. However, these patients showed different exon 10 (P6) and exon 13 (P7) SSCP patterns (Fig. 4a), which were not found in controls and unrelated subjects. These patterns were ascribed to the silent mutations V202V (caused by a C-to-T transition at position 606 (data not shown)) and A266A (caused by a substitution

Fig. 3. Mutation analysis of exons 12 and 15 of the PBGD gene. **A:** PCR-SSCP analysis of exon 12: (lane 1) normal control; (lanes 2 and 3) abnormal pattern (asterisk) observed in the patient P3 and one relative. **B:** Direct sequencing (antisense strand) showed a C-to-T transition at position 673 of the coding sequence. **C:** PCR-SSCP analysis of exon 15: (lane 1) normal control; (lanes 2–4, and 6) unrelated AIP patient with no mutation in this region; (lane 5) abnormal SSCP pattern (asterisk) displayed by patient P4. **D:** Direct sequencing (antisense strand) showed a C-to-T transition at the position 973 corresponding to the coding sequence.

of C-to-T at position 798) (Fig. 4b). The silent mutation A266A observed in this study has not been reported heretofore.

Polymorphisms

In a comparison of patients and controls, the amplified fragments containing exons 4 and 11 showed different patterns of SSCP, suggesting the occurrence of polymorphisms. Three specific SSCP patterns were associated to homozygote CG, homozygote AA, and heterozygote GA. The amplified fragment containing

exon 11 showed four patterns in the SSCP analysis, but the association to the genotype was difficult to analyze because two adjacent polymorphisms (7064 C/A and 7052 G/A) were detected. In addition, direct sequencing of the amplified fragments containing exons 1 and 3 revealed the polymorphisms –65 C/T and 3119 G/T, respectively.

DISCUSSION

The aim of this study was to investigate the occurrence of causative mutations in unrelated Brazilian

(A) DNA - SSCP (Exon 13)

(B) DNA silent mutation: A266A (798 G → A)

Fig. 4. Mutation analysis of exon 13 of the PBGD gene. **A:** PCR-SSCP analysis of exon 13: (lanes 1–5) normal control and four unrelated AIP patients with no mutation in this region; and (lane 6) abnormal pattern (asterisk) displayed by patient P6. **B:** Direct sequencing (antisense strand) revealed a G-to-A transition at position 798, causing the silent mutation A266A.

AIP patients. Mutations in the 15 exons, exon-intron junctions, and the housekeeping promotor region of the PBGD gene were investigated by PCR-SSCP and direct DNA sequencing analysis. This approach has been recommended because the genetic alterations may occur throughout the entire gene and they usually are family-specific (13,14). The sensitivity of SSCP analysis for detecting PBGD gene mutations was previously studied in Finnish AIP patients (12). Using a combination of different temperature and glycerol concentrations in SSCP, 17 out of 19 mutations (89%) were detected; the best match was a gel run without glycerol at 4°C. Other screening methods based on heteroduplex analysis (15), denaturing gradient gel electrophoresis (DGGE) (13),

TABLE 1. Summary of the PBGD gene mutations identified in seven acute intermittent porphyria (AIP) patients from Brazil

Patients	Exon	Nucleotide change	Location	Mutation
P1	7	G to A	331	G111R
P2	9	C to T	445	R149X
P3	12	C to T	673	R225X
P4	15	C to T	973	R325X
P5	7	G to A	331	G111R
P6a	10	G to T	606	V202V
P7a	13	G to A	798	A266A

*Silent mutations probably not related to AIP phenotype.

and chemical cleavage of mismatch (CCM) (14) are also useful for the detection of abnormalities in the PBGD gene.

In the present study, four different mutations, which have already been described in AIP patients (Table 1), and five polymorphisms were identified. Four AIP-related mutations were found in five patients, and two silent mutations were revealed in patients P6 and P7. As these silent mutations do not lead to alterations in the PBGD protein, further investigation will be necessary to define the mutations responsible for the disease in these patients.

Three out of four mutations detected in our AIP patients were nonsense mutations leading to premature stop codons. The mutation R149X reported herein, which leads to a substitution of arginine¹⁴⁹ by a stop codon due to a C-to-T nucleotide transition at position 445, was first identified in Finland (12). Studies on enzyme tertiary structure (17) revealed that several highly conserved arginine residues were directly involved in important functions, such as initial substrate binding, extension of the pyrrole chain, and tetrapyrrolic product release. This mutation leads to a truncated protein, which probably results in an inactive enzyme.

A C-to-T transition was identified in exon 12, which replaced an arginine for a termination signal (CGA-to-TGA) at codon 225 (R225X). This transition was previously described in Finland (12); it was also detected by direct solid-phase sequencing in a Northern European family (18), and by DGGE analysis in a North American patient (19). A dramatic decrease (fivefold) in the amount of the mutant transcripts has been observed in three different members of a family who carry this nonsense mutation (20). The nonsense mutation R325X was caused by a C-to-T transition at position 973 in exon 15. This mutation was originally described by Petersen et al. (21) in Danish families, and has also been found in a French AIP family (13). These nonsense mutations may lead to a nonfunctional or unstable PBGD protein.

The G111R mutation was caused by a G-to-A transition at position 331, leading to a glycine-to-arginine substitution. Originally observed in Europe, this amino acid change at the surface of protein has been shown to lead to an inactive protein, as demonstrated by site-directed mutagenesis and expression studies (22). The mutation was also reported in Slavic (23), French (13), British (24), and Swedish (10) patients. Recently, it was detected in 12 Argentinian AIP families, suggesting a founder effect (11).

More than 130 AIP causative mutations on the PBGD gene have been reported (10), and molecular analysis has been used with confidence to detect carriers and define diagnoses. Mutation detection in AIP families enables the identification of latent porphyria carrier and noncarrier members. The exact AIP non-carrier assignment avoids preventive measures usually prescribed for carriers, and makes it unnecessary to screen their offspring. The identification of AIP carriers is of clinical importance and may be useful in the avoidance of known triggering agents.

In conclusion, genomic DNA screened by 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 to the previously reported AIP causative mutations, intragenic polymorphisms -65 C/T, 3119 A/G, 3581 G/A, 7052 G/A, and 7064 C/A were detected. Two silent mutations, V202V and A266A (which was previously undetected), were also observed.

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