Molecular Diagnosis of Acute Intermittent Porphyria by Analysis of DNA Extracted from Hair Roots

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Analysis for mutations in the porphobilinogen deaminase gene offers a more definitive diagnosis of acute intermittent porphyria (AIP) than do conventional biochemical tests. We used single-strand conformation polymorphism analysis followed by direct sequencing to identify a new G → A mutation at the last position of intron 7 in a patient with AIP. The mutation disrupts the invariant AG dinucleotide at the 3′ splice acceptor site and therefore interferes with mRNA processing. To identify other individuals who inherited this mutation, we analyzed five hairs with intact roots collected by each participating family member and sent to us by mail. DNA was extracted from the hair roots and amplified by the polymerase chain reaction. The amplified products were digested with the restriction enzyme BsaI to confirm the presence or absence of the mutation. All six family members who were known to have AIP tested positive, as did three members who had not been previously diagnosed. Hair roots provide a convenient, accessible, and economical alternative to blood as a source of DNA for molecular diagnostic testing.

Indexing Terms: porphobilinogen deaminase/single-strand conformation polymorphism/splice junction mutation/porphyria/genetic disorders/metabolic disorders

Acute intermittent porphyria (AIP) is a hereditary disorder of porphyrin metabolism caused by a partial deficiency of porphobilinogen (PBG) deaminase (EC 4.3.1.8), the third enzyme in the heme synthetic pathway (1). 4 The inheritance pattern is autosomal dominant. Patients with AIP have attacks of abdominal pain, constipation, vomiting, neurological signs and symptoms, and psychiatric disturbances. Attacks can be triggered by consumption of alcohol, certain drugs and hormones, caloric deprivation, and other factors. Only a fraction of gene carriers are clinically affected (2), and the most important aspect of patient management is avoidance of factors that may precipitate an attack. This requires a diagnostic strategy that accurately identifies asymptomatic gene carriers as well as those with clinically expressed disease.

Mutational analysis of the PBG deaminase gene offers a definitive means of identifying patients who have inherited the genetic defect. To date, >30 mutations causing AIP have been reported. About half of the mutations have been found in exon 10 (3-10) and exon 12 (4, 8, 11-13), and most of the others have been located in exon 1 (14), exon 4 (8, 13), exon 5 (13), exon 6 (13), exon 8 (5), exon 9 (4, 10, 13, 15), and exon 14 (4). The majority of these mutations are single base-pair substitutions in genomic DNA; the remainder are due to small insertions or deletions. Point mutations that alter splice junction sites in intron 1 (16), intron 5 (13), and intron 9 (9) have also been described. Once a mutation has been defined, other individuals who have inherited it can be identified by restriction enzyme digestion (6), hybridization to allele-specific oligonucleotides (16), or allele-specific amplification (9).

Testing other family members for the mutation requires a source of genomic DNA. Whole blood is the most commonly used specimen for genetic testing, but blood collection is an invasive procedure, and asymptomatic individuals may be unwilling to have their blood drawn. Blood collection also involves the inconvenience and expense of traveling to a doctor’s office or clinical laboratory. When the patient lives at a distance from the laboratory where the test is performed, there is an additional cost for sample transport, handling, and storage. An alternative source of DNA for genetic testing is the use of hair roots (17, 18). Collection of hair samples is noninvasive and relatively painless, can be performed by the patient or a family member, obviates the need for special collection and handling procedures, and eliminates nearly all of the associated transportation costs.

We have recently identified a new mutation in the PBG deaminase gene that alters the 3′ splice acceptor site preceding exon 8. To diagnose family members living in several locations throughout the US, we sent them instructions on how to collect and send hair samples to our laboratory for analysis. Here we describe the protocol used to identify the mutation and the results of genetic testing performed on DNA extracted from hair roots.

Materials and Methods

Patients and control subjects. Whole blood was collected by venipuncture into heparin-containing tubes, and the erythrocyte fraction was isolated by centrifugation. The diagnosis of AIP was confirmed by measurement of PBG deaminase activity and immunoreactive concentration in erythrocyte lysates (19). For the index case, enzyme activity was 71 pkat/g hemoglobin and the immunoreactive concentration was 90 μg/g hemoglobin;

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4 Nonstandard abbreviations: AIP, acute intermittent porphyria; PBG, porphobilinogen; SSCP, single-strand conformation polymorphism; PCR, polymerase chain reaction; cDNA, complementary DNA.

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the corresponding mean values for nine controls were 140 pkat/g hemoglobin and 181 μg/g hemoglobin. The specific activity of PBG deaminase, calculated as enzyme activity divided by immunoreactive concentration, was similar to control values, indicating a cross-reacting immunological material-negative form of AIP. Procedures involving human subjects were performed in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Screening for the mutation. Single-strand conformation polymorphism (SSCP) analysis (20) was performed on all 15 exons of the PBG deaminase gene. Genomic DNA was extracted from leukocytes obtained from EDTA-anticoagulated whole blood in a Model 341 DNA Extractor (Applied Biosystems, Mississauga, ON). Amplification of genomic DNA by the polymerase chain reaction (PCR) was carried out in a total volume of 50 μL containing, per liter, 10 mmol of Tris-HCl (pH 8.3), 1.5 mmol of magnesium chloride, 50 mmol of potassium chloride, 0.1 g of gelatin, 25 kU of Taq polymerase, 200 μmol of each deoxynucleotide, 1 μmol of each primer, and 5 mg of genomic DNA. Each 50-μL reaction also contained 12.5 μCi of [α-thio-35S]dATP. After an initial denaturation step at 94°C for 5 min, samples were run through 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min in a DNA Thermal Cycler (Perkin-Elmer Canada, Rexdale, ON). All exons were amplified under these conditions. One volume of each PCR product was added to one volume of a solution of 950 mL formamide, 20 mmol/L EDTA, 1 g/L sodium dodecyl sulfate, 0.5 g/L bromphenol blue, and 0.5 g/L xylene cyanol. The mixture was heated at 96°C for 2 min and then placed on ice. Samples (3 μL) were loaded onto a 6% polyacrylamide gel containing 50 mL/L glycerol and run at 60 W for 4–6 h at room temperature, with use of a fan to keep the gel cool. Gels were dried on filter paper, then exposed to x-ray film for 1–3 days before developing the autoradiogram. Banding patterns were inspected visually for mobility changes in single- and double-stranded DNA. Each exon was analyzed at least twice, once intact and once or twice after digestion with a restriction enzyme, to increase the likelihood of finding a sequence abnormality.

The primer sequences and the sizes of the PCR products they generate have been described (10). Primers were designed to amplify each exon and at least 10 to 20 bases of flanking intronic sequence.

Sequencing of exon 8. Exon 8 and its flanking intronic sequences were amplified by using primers 20 (5′ CAGTGTGGGGGCAATGAG, sense strand) and 21 (5′ TGGCTCTTCTGGGCACTCC, antisense strand). The PCR product was precipitated with 4 mol/L ammonium acetate:isopropanol (1:2, by vol), washed with cold 800 mL/L ethanol, and redissolved in 12 μL of 10 mmol/L Tris buffer containing 1 mmol/L EDTA (pH 8.0). One microliter of the redissolved product was used as template in a second PCR, which was performed in duplicate under identical conditions except that the concentration of either the forward or reverse primer was decreased by 100-fold. The products were precipitated, washed, and redissolved as for the first PCR. Direct sequencing of both amplified strands was performed by the dye primer termination method (Sequenase sequencing kit; United States Biochemical, Cleveland, OH) with the PCR primers as sequencing primers.

Complementary DNA (cDNA) analysis. The patient’s lymphocytes were isolated by centrifugation in a gradient of Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden), and total RNA was extracted by treatment with guanidinium hydrochloride and density-gradient centrifugation in CsCl. cDNA was prepared by reverse transcription with an oligo dT primer (cDNA cycle kit; Invitrogen, San Diego, CA). The cDNA for PBG deaminase was amplified by PCR with primers 36 (5′ AGC-GAATTCACTGCTCTGTAACGGCAA, sense strand) and 37 (5′ CCCAAGCTTCTGTGCCCCACAAACCA, antisense strand), which encompass the entire coding sequence. Reagent concentrations were as described above, and a 30-cycle program of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s was used. Primer 36 contains an EcoRI restriction site and primer 37 contains a HindIII restriction site for cloning the cDNA. The size of the amplified cDNA was analyzed by both agarose and polyacrylamide gel electrophoresis. The gel-purified cDNA was digested with EcoRI and HindIII, ligated into the corresponding sites of a Bluescript vector (Stratagene Cloning Systems, La Jolla, CA), and cloned into a DH5α strain of Escherichia coli. Plasmids were isolated by an alkaline lysis procedure and sequenced in the reverse direction by the dye primer chain termination method, by using primer 6 (5′ TTTCTCTGAGCTGGGCTGC, antisense strand), which anneals to exon 9, as a sequencing primer.

Hair samples. Family members were sent instructions to pull five hairs from their own scalp, such that each hair shaft had a visible follicle at the end. All hairs were to be placed directly into a clean envelope that the individuals would seal themselves. If it became necessary to collect another person’s hair (e.g., from children), clean plastic or latex gloves were to be worn and the envelope sealed with tape, not by licking. Hair that was too long could be cut with scissors, but the follicle was not to be allowed to contact other surfaces, to prevent possible contamination. Hair samples were mailed to our laboratory by first-class mail and stored at room temperature until analysis.

Extraction and amplification of DNA. Hair shafts were removed with a sharp blade, and two follicles from each individual were placed in a 0.5-mL microcentrifuge tube. Five microliters of 10-fold-concentrated PCR buffer [100 mmol/L Tris-HCl (pH 8.3), 15 mmol/L magnesium chloride, 500 mmol/L potassium chloride, 1 g/L gelatin], 5 μL of proteinase K (1 g/L), and 26.5 μL of water were added, and samples were incubated at 60°C for 30 min and then at 100°C for 10 min. We next added 8 μL of deoxynucleotide triphosphates (1.25 mmol/L), 2.5 μL each of primers 20 and 21 (20 μmol/L), and 0.25 μL of Taq polymerase (5000 kU/L) to each sample and
carried out 30 cycles of PCR, using the temperature cycling program described for amplifying exon 8. One microliter of this mixture was reamplified in a second round of PCR with the same primers.

Electrophoretic analysis. PCR samples (6 μL) were electrophoresed on Visigel Separation Matrix (Stratagene) at 10 V/cm for 1 h and then stained with ethidium bromide for 0.5 h to verify the presence of the expected 213-bp product corresponding to exon 8 and its flanking intronic sequence. Samples were then digested with BsaJI (New England Biolabs, Mississauga, ON) for 2 h at 60°C (12 μL of sample, 2 μL of BsaJI, 2 μL of 10× buffer, and 4 μL of water), and the digest was electrophoresed on Visigel Separation Matrix. Two smaller fragments of 132 and 81 bp were present in unaffected subjects; family members with the mutant allele showed a third band at 213 bp, corresponding to uncut PCR product.

Results

SSCP analysis was performed on the index case, 12 other patients with AIP from 8 unrelated families, and 15 individuals with normal PBG deaminase activity. Samples were run on the same gel to facilitate comparison of the banding patterns. The index case showed an extra band in exon 8 that was not present in other samples. A second run after digestion of samples with BsaJI also showed an extra, apparently undigested, band for the patient. No other nonpolymorphic banding patterns were noted for this patient on SSCP analysis of the other 14 exons.

Direct sequencing of exon 8 after asymmetric PCR revealed both a guanine and adenine at the last position of intron 7; no other sequence abnormalities were found (Fig. 1). Because AIP is an autosomal dominant disorder, one mutant and one normal allele should be present. The AG dinucleotide is an invariant feature of 3′ splice junctions (21), and changes in base would be expected to cause a defect in splicing of the primary RNA transcript. To investigate the effect of the mutation on RNA processing, we reverse-transcribed the patient’s total RNA and amplified the coding sequence for PBG deaminase by PCR. Analysis of the product on both agarose and polyacrylamide gels showed a single band, suggesting that the mutation did not cause exon skipping. The PCR product was cloned, and 12 clones were sequenced to identify the junction of exons 7 and 8 in the cDNA. In all clones, only the normal sequence was detected, corresponding to the normal allele. Apparently, neither exon skipping nor activation of a cryptic splice site is occurring, and the RNA transcript from the mutant allele may be too unstable to identify.

The G → A transition at the 3′ splice junction disrupts the recognition sequence for BsaJI (CCNNGG), providing a rapid means of testing for the defect. The PCR product containing exon 8 is 213 bp long, and digestion with BsaJI generates two smaller fragments of 132 and 81 bp. The patient and much of her extended family live about 1000 miles from our laboratory, and some family members live in other, more distant locations. The patient arranged for hair samples from family members to be collected according to our instructions and sent to our laboratory. Extraction of DNA and first-round PCR generated products that were occasionally indistinct and hard to visualize on gels (Fig. 2). A second PCR followed by electrophoresis showed clear, strong bands of the expected size, corresponding to exon 8 and its flanking intronic sequence. Digestion with BsaJI produced two smaller fragments of 132 and 81 bp in unaffected individuals. Family members with the mutant allele showed a third band at 213 bp, corresponding to uncut PCR product (Fig. 3).

Of 37 individuals tested (including several spouses), 9 family members were positive, including all those who had been previously diagnosed with AIP. Several family members who had not displayed symptoms or been
The **G** → **A** mutation at the last position of intron 7 is one of four intronic mutations that cause AIP. Another **G** → **A** mutation at the last position of intron 9 also disrupts a 3’ splice acceptor site (9), and **G** → **A** mutations at the first positions of intron 1 (16) and intron 5 (13) interfere with the respective 5’ splice donor sites. In only one of these cases was an mRNA of abnormal size demonstrated (13). However, all four mutations affect invariant sequences in the donor and acceptor splice sites, which would prevent the normal processing of mRNA and presumably result in failure to produce a functional enzyme from that allele (21, 24).

The use of hair roots as a source of DNA for family studies enabled us to obtain samples from several locations throughout the US without the expense and inconvenience associated with blood collection. Since PCR requires only very small amounts of target sequence, we found that hair roots provided sufficient template for amplifying the area of interest. The first-round PCR generated a product that was often too weak for subsequent analysis, in part because of inhibition of the reaction by hair root constituents released during DNA extraction. A second PCR gave a strong band at 213 bp for all samples (Fig. 2). Digestion with the restriction enzyme BsuJI distinguished gene carriers from family members who did not inherit the mutation (Fig. 3).

We received blood samples from only two patients, the index case and an affected relative. In both patients, the mutation was present in DNA obtained from leukocytes and hair roots. Given that previous work has validated the use of hair roots for genetic testing (18), we did not consider it necessary to confirm the results of other family members by analysis of leukocyte DNA. Despite the simple specimen collection and transport procedure, hair root samples were never received from one affected individual (II-3; Fig. 4) or from five members at risk for inheriting AIP (III-5, III-7, III-11, IV-11 and IV-12). Had we requested blood samples from all family members, the response rate would probably have been much lower.

In summary, we have demonstrated that molecular diagnostic testing for AIP can be performed on hair roots. This approach to sample collection is noninvasive and eliminates problems associated with blood collection and transport, especially when individuals who need testing live in widely separate geographic locations. It also involves patients in their own medical care, which may increase their willingness to be tested and to respond positively to the results of those tests.

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References


Discussion

The biochemical diagnosis of AIP is based on increased excretion of δ-aminolevulinic acid and porphobilinogen in urine and a decrease in the activity of PBG deaminase in erythrocytes. Asymptomatic gene carriers may excrete normal amounts of these porphyrin precursors, and the enzyme assay can be ambiguous because of the overlap in values between affected and unaffected individuals (22). Linkage analysis with polymorphic markers in the PBG deaminase gene has been used to identify gene carriers (23), but this technique is not always informative. DNA analysis for the causative mutation offers the most accurate means of identifying gene carriers. Many different mutations are known to cause AIP, so a screening method for finding the mutation in a given family is necessary. We have used SSCP to identify the exon of interest, followed by direct sequencing to precisely define the mutation. Denaturing gradient gel electrophoresis has also been successfully applied to the detection of mutations that cause AIP (7, 13). The causative nature of the mutation is inferred from its effect on mRNA processing and (or) protein structure, its segregation with the disease, and its absence in control specimens.

![Fig. 3. BsaJ I digest of exon 8 resolved on Visigel Separation Matrix.](image)

In unaffected individuals, digestion with BsaJ I produces two fragments of 132 and 81 bp (lanes 3, 6, and 7). The point mutation in intron 7 destroys the BsaJ I site, causing a band of 213 bp to appear in gene carriers (lanes 2, 4, and 5). Note that BsaJ I does not completely cut the PCR product, and the relative intensities of the three bands must be taken into account when interpreting the patterns. Lane 1: size markers (700–100 bp in 100-bp increments).

Fig. 4. Pedigree showing the results of molecular testing for the G → A mutation in intron 7.

Fig. 4 Pedigree showing the results of molecular testing for the G → A mutation in intron 7. (□) Males; (□) females; (□), AIP (tested positive); (+), previously undiagnosed AIP (tested positive); (□), no AIP (tested negative); (X), not tested; (·), deceased. Patients I-2 and I-3 were previously diagnosed with AIP.

tested by conventional laboratory means were also identified as gene carriers. A pedigree of the family is shown in Fig. 4.

[Image of a pedigree chart showing genetic testing results.]

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References