Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria

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Mutation analysis of genomic DNA samples obtained from 17 unrelated South African patients with variegate porphyria (VP) revealed three novel missense mutations in the protoporphyrinogen oxidase (PPOX) gene. A common C to T transition at nucleotide position 452 (R59W) was identified in 15 of the patients analysed, while base changes at positions 336 (H20P) and 779 (R168C) were identified in the remaining two patients. Using protein analysis software we were able to predict that all three mutations have a similar biophysical effect on the protein, being the disturbance of amphiphatic regions within the protein, which might result in misfolding of the protein. Mutation R59W, identified in the majority of South African VP families, was shown to create a Sty I restriction site, while mutation R168C would abolish a Dsa I restriction site in genomic DNA of affected individuals. As 100% of the index patients analysed were molecularly characterized, the combined use of restriction enzyme and single-strand conformation polymorphism (SSCP) analysis now allows a rapid and accurate diagnosis of VP in South Africa. Mutation R59W was furthermore shown to be in association with one of four potential haplotypes defined by two newly described polymorphisms in exon 1 of the PPOX gene. Our molecular data thus strongly support the founder hypothesis for VP in South Africa.

INTRODUCTION

Variegate porphyria (VP), one of the acute hepatic porphyrias, is an autosomal dominant disease characterized by a mean 50% reduction in the activity of protoporphyrinogen-IX oxidase (PPOX, EC 1.3.3.4), the seventh enzyme in the pathway of haem biosynthesis. PPOX is located in the inner mitochondrial membrane (1) where it enzymatically oxidases protoporphyrinogen to protoporphyrin.

VP is associated with cutaneous manifestations, including photosensitivity, attacks of abdominal pain and neuropsychiatric dysfunctions, and an overexcretion of coproporphyrin and protoporphyrin in urine and stools. The disease has a very high prevalence in South Africa (0.003) compared with the rest of the world and this has been ascribed to a founder effect (2).

Both cDNA and genomic DNA clones of the human PPOX gene have been isolated and from genomic clones it has been deduced that the gene spans about 8 kb and contains 13 exons (3,4). Recently, two mutations in the PPOX gene were identified in VP families of French Caucasian origin, providing evidence that VP is caused by defects in this gene (5). In this study single- (SSCP) and double-strand (heteroduplex) conformation polymorphism analyses were used (6,7) to screen all 13 PPOX gene exons, including the splice site sequences, for mutations in 17 unrelated South African VP patients. A common single base substitution was found in 88% of cases, while two different mutations were identified in the remaining two index patients. In addition, two polymorphisms were detected in the first untranslated exon of the PPOX gene. Association observed between the mutation identified in the majority of patients analysed and one of four potential haplotypes as defined by two exon 1 polymorphisms, strongly supports the founder hypothesis for VP in South Africa.

RESULTS

A total of 17 VP patients and 10 normal controls were included in the initial DNA screen of all 13 PPOX exons. Sequences of 12 oligonucleotide primer pairs designed for polymerase chain reaction (PCR) amplification of individual exons and limited flanking intron sequences of the PPOX gene are given in Table 1. Heteroduplex–SSCP analysis of PCR products generated by using these primers indicated the presence of mutations in exons 1, 2, 3 and 6.
Table 1. Primers used for PCR, SSCP and sequencing analysis of the PPOX gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>PCR product size (bp)</th>
<th>Amplified region</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO1F</td>
<td>CCGCCAATCAGACATGAGGAG</td>
<td>327</td>
<td>Exon 1</td>
</tr>
<tr>
<td>PPO1R</td>
<td>TTCCCGCTGACAGGTCGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPO2F</td>
<td>CTTTGAGGAGGAGCAGTGTGTC</td>
<td>185</td>
<td>Exon 2</td>
</tr>
<tr>
<td>PPO2R</td>
<td>TAAATGAAGGCTCCTCCCTGGC</td>
<td>234</td>
<td>Exon 3</td>
</tr>
<tr>
<td>PPO3F</td>
<td>TGCACTGCTCTCCCTCTTGT</td>
<td>180</td>
<td>Exon 4</td>
</tr>
<tr>
<td>PPO3R</td>
<td>GCTCCCTAATCCTATTC</td>
<td>247</td>
<td>Exon 5</td>
</tr>
<tr>
<td>PPO4F</td>
<td>GAGGCTGAGGAGGAGTCAGG</td>
<td>237</td>
<td>Exon 6</td>
</tr>
<tr>
<td>PPO4R</td>
<td>TTAATGAAGGCTCCTCCCTGGC</td>
<td>272</td>
<td>Exon 7</td>
</tr>
<tr>
<td>PPO5F</td>
<td>TGCAGTGTCTCTCCCTCTTGT</td>
<td>197</td>
<td>Exon 8</td>
</tr>
<tr>
<td>PPO5R</td>
<td>GTACGTTCAATCCTCTCAGG</td>
<td>202</td>
<td>Exon 9</td>
</tr>
<tr>
<td>PPO6F</td>
<td>CGGTATGCTGACAGGAGGCTCTGG</td>
<td>223</td>
<td>Exon 10</td>
</tr>
<tr>
<td>PPO6R</td>
<td>TGTGAGGACTGTCAGACATTG</td>
<td>257</td>
<td>Exon 11</td>
</tr>
<tr>
<td>PPO7F</td>
<td>CTTTGAGGAGGAGCAGTGTGTC</td>
<td>333</td>
<td>Exon 12,</td>
</tr>
<tr>
<td>PPO7R</td>
<td>TAGAACGACCAGGACAGGAC</td>
<td></td>
<td>intron 12 and exon 13</td>
</tr>
</tbody>
</table>

Analysis of exon 3 of the PPOX gene revealed aberrant mobility SSCP and heteroduplex bands in 15 VP patients. These band shifts are shown in Figure 1A in one of the VP patients (lane 2), compared to the banding pattern in a normal control (lane 1). Sequencing of exon 3 PCR products in patients and controls revealed a C → T at position 452 base with similar intensities in VP patients, but only a C base at this position in normal controls which is in agreement with the published cDNA sequence (3). This mutation, designated R59W, would result in an arginine to tryptophan amino acid change. From the published sequence it was also predicted that the sequence change would cause an extra StyI restriction site. As expected, digestion of exon 3 PCR products resulted in 138 and 96 bp fragments in normal individuals, while the larger DNA fragment was cut into two fragments of 112 and 26 bp in mutated alleles (Fig. 1B). With the aid of this rapid screening test, we were able to confirm that mutation R59W segregates with the disease in all 15 VP families (a total of 43 family members) from which the initial 15 patients originated. The exon 3 mutation was absent in 80 normal chromosomes screened and is therefore not likely to be a neutral polymorphism.

The two remaining index patients showed aberrant SSCP patterns in the amplification products of exons 2 and 6, respectively (Fig. 2A, B). Sequence analysis in these VP patients and normal controls revealed an A336→C336 transversion in exon 2, and a C779→T779 transition in exon 6 (Fig. 2C, D). DNA of an affected family member of each index case was also sequenced and independently confirmed these findings. These apparently infrequent base changes identified in single South African VP families are both missense mutations, causing amino acid changes from histidine to proline (H20P) and arginine to cysteine (R168C), respectively. The R168C mutation abolishes a cleavage site for the restriction enzyme DsaI, while the H20P mutation does not alter any known restriction enzyme cutting site. Owing to the cost of DsaI, the two mutations were traced in the respective families by SSCP analysis. Both mutations co-segregate with VP and were absent in 80 normal chromosomes screened.

Two additional point mutations were identified at nucleotide positions 26 and 150 in exon 1 of the PPOX gene (data not shown) after comparison with published sequence data (3, 4). Both these mutations are likely to represent neutral polymorphisms as they were observed in VP patients as well as normal controls. Among the normal and affected South African subjects analysed, the newly described C26→G26 allele was more common than the A26 allele reported at this position in the PPOX gene (4). The allele frequencies for this polymorphism, designated I-26, were 0.35 for the A26 allele and 0.65 for the C26 allele among 40 Afrikaners. The second polymorphism identified as a C150→G150 mutation (I-150), destroys a BanII cutting site and is relatively rare with allele frequencies of 0.95 and 0.05, respectively. The frequencies of the rare alleles were lower among VP patients (0.18 and 0.03 for polymorphisms I-26 and I-150, respectively), but these differences were statistically non-significant for the standard contingency Chi-square test conducted on the raw data. Genotyping of the two exon 1 polymorphisms in VP family members have shown that mutations R59W and H20P are associated with haplotype C26,C150, while mutation R168C is associated with haplotype A26,C150.
combined heteroduplex–SSCP analysis was used to screen the To investigate the molecular basis of VP in South Africa, DISCUSSION

Figure 1. Detection of mutation R59W by heteroduplex-SSCP and restriction enzyme analysis. (A) Combined heteroduplex-SSCP analysis in a 10% PAA gel supplemented with 7.5% urea. Lane 1, PCR product of a normal control; lane 2, PCR product of a VP patient showing both an SSCP shift (upper bands) and heteroduplex formation (lower bands). (B) Co-segregation of VP and mutation R59W in an affected family. Half-darkened pedigree symbols indicate clinically diagnosed VP patients. Exon 3 PCR products were digested with StyI and electrophoresed in a 12% PAA gel. The index case (lane 1) and her affected daughter (lane 3) show extra bands of 112 bp after StyI digestion while the unaffected spouse (lane 4) and son (lane 2) show only the expected 138 and 96 bp DNA fragments. A DNA molecular size marker was loaded in lane 5.

Figure 2. Identification of two point mutations in the PPOX gene by SSCP analysis and DNA sequencing. SSCP were detected in exons 2(A) (lane 2) and 6 (B) (lane 2) of the PPOX gene in two VP patients. PCR-amplified DNA of a normal control is shown in the first lanes on the 20% PAA gels. Direct sequencing of PCR products revealed that the patients are heterozygous for mutations at nucleotide positions 336 (C) and 779 (D), respectively.

DISCUSSION

To investigate the molecular basis of VP in South Africa, combined heteroduplex–SSCP analysis was used to screen the entire coding region systematically as well as the exon–intron boundaries of the PPOX gene for mutations in 17 unrelated VP patients. A frequently occurring exon 3 mutation, R59W, was characterized in 88% of South African VP patients, while less frequent mutations, H20P and R168C, were identified in exons 2 and 6, respectively, in single families.

The R59/168 and H20 residues affected by the mutations described in this paper are all evolutionarily highly conserved (8) and are therefore believed to be of importance for proper functioning of the protein. In order to predict the possible effects of the various mutations on the protein characteristics, we compared wild-type and mutated primary protein sequences using the Protean program present in the Lasergene (DNASTR Inc.) software package. Comparison of several characteristics, including hydropathy, secondary structure, antigenicity, amphility, flexibility, charge-density and surface probability, indicated that besides the hydrophobic to hydrophilic (and vice versa) changes which could already be predicted solely based on the physical characteristics of the amino acids in question, in all three cases, amphiphatic patterns (alpha-amphiphatic regions in the case of the H20P and R168C mutations, beta-amphiphatic regions in the case of the R59W mutation) within the wild-type protein were altered. Amphiphatic regions are helices described by one polar and one apolar face (9), and are considered to play an important part in the maintenance of the protein structure. Additional experiments are required to investigate whether a general misfolding of the protein or specific alterations within possible interacting domains are causing the reduction in function of the various mutated forms of the protein.

If there was a single common ancestor for VP in South Africa, as previously suggested (2), one mutation would be expected to predominate in the present population. The high frequency of mutation R59W found among South African VP patients is thus in accordance with the founder hypothesis. It is well known that methylated CG sequences are prone to deamination of C\textrightarrow\text{T} (10), but the additional finding that this mutation is always associated with a common haplotype defined by two exon 1 PPOX gene polymorphisms, excludes the possibility of recurrent mutational events at the CpG hotspot spanning codon 59. A search for additional polymorphisms in and around the PPOX gene to extend the haplotype analysis in families with mutation R59W will further complement the classical assessment of a founder effect for VP in South Africa.

The identification of PPOX gene mutations in VP families is at present a major goal both for genetic counselling and for understanding additional mechanisms of the disease. The variable clinical expression of VP within and between families with the same PPOX gene mutation (11) requires further investigation to elucidate genotypic and phenotypic relationships. Knowledge of the gene mutations underlying VP in South Africa now enables rapid molecular diagnosis of the disease, obviating the need to rely on biochemical assays and clinical symptoms.

MATERIALS AND METHODS

Subjects

A total of 17 VP patients unrelated to the second degree, 106 of their family members and 40 normal controls were the subjects of this study. Clinical diagnoses of VP for the families studied were available from a previous study (12). The normal controls...
were from the Afrikaner population, as it was in this population that a high incidence of VP was originally described (2).

**Mutation analysis**

Genomic DNA was amplified by PCR in a total volume of 50 μL containing 50 ng DNA, 20 pmol of each primer, 200 μM dNTPs, 0.5 U Taq polymerase and 1×buffer (Boehringer Mannheim). The amplification profile was 60 s at 95°C, followed by 10 cycles at 95°C for 30 s, 65°C for 45 s, 72°C for 30 s, and 30 cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 30 s, with a final extension at 72°C for 8 min. All 13 exons were amplified using these conditions and oligonucleotide primers (Table 1) designed from intrinsic sequences (S. Taketani, unpublished data).

The PCR-amplified DNA was denatured at 95°C for 4 min and loaded on to low cross-linked (1% C) urea supplemented 10% polyacrylamide (PAA) gels for combined heteroduplex–SSCP analysis (13). Two additional gel systems, 10% PAA gels supplemented with 5% glycerol and 20% PAA gels, electrophoresed at room temperature and 4°C overnight, respectively, were used for SSCP analysis. A PCR template preparation kit (Pharmacia Biotech) was used to prepare single-stranded DNA from PCR products of patients showing abnormal electrophoretic patterns and of normal controls. DNA sequence analysis was performed with the T7 sequencing kit (Pharmacia Biotech). One of the mutations (R59W) identified in the PPOX gene introduces a StyI cutting site, and 16 μl of each PCR reaction were digested overnight with 20 U StyI (Boehringer Mannheim). The digested exon 3 PCR products were electrophoresed on a 12% PAA gel, stained with ethidium bromide and photographed under ultraviolet light.

**Polymorphism analysis**

All study participants were genotyped for two polymorphic sites identified in exon 1 of the PPOX gene, using SSCP and/or BanII (I-150) restriction enzyme analysis. VP-associated haplotypes were deduced by segregation analysis, based on the assumption that there were no recombination events within families. Allele frequencies were determined by allele counting. χ² tests were performed on the control population to investigate possible departures from Hardy–Weinberg equilibrium frequencies for the two polymorphisms.

**ABBREVIATIONS**

VP, variegate porphyria; PPOX, protoporphyrinogen oxidase.

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**REFERENCES**