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RESEARCH ARTICLE

Identification and Characterisation of a Deletion (537delAT) in the Protoporphyrinogen Oxidase Gene in a South African Variegate Porphyria Family

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Variegate porphyria is an autosomal dominant disorder of haem metabolism resulting from a partial decrease in protoporphyrinogen oxidase activity. Variegate porphyria is highly prevalent in South Africa, the result of a founder effect now confirmed genetically as a single point mutation (R59W) which has been described in nearly all South African variegate porphyria patients studied. Only two other mutations (H20P, R168C) have been reported in South Africa. We utilised simultaneous, single-stranded conformational polymorphism and heteroduplex analysis, and direct sequencing to identify a further mutation; a 2 bp deletion in exon 6 which results in a premature stop codon 11 codons downstream from the mutation and is the first reported deletion in the protoporphyrinogen oxidase gene in a South African family. The familial segregation of this mutation strongly suggests that it is the disease causing mutation for variegate porphyria in this family. This further evidence for allelic heterogeneity limits the utility of tests for the R59W mutation in the diagnosis of variegate porphyria in South Africa. Hum Mutat 12:403–407, 1998.

KEY WORDS: **protoporphyrinogen oxidase; variegate porphyria; porphyria; haem synthesis; deletion**

INTRODUCTION

Variegate porphyria (VP) is an autosomal dominant disorder of haem metabolism with incomplete penetrance and results from decreased activity of the haem synthetic enzyme protoporphyrinogen oxidase (PPO; EC 1.3.3.4; MIM # 600923) (Brenner and Bloomer, 1980; Deybach et al., 1981; Meissner et al., 1996). The disease is characterised by photocutaneous sensitivity and a propensity to develop acute and potentially fatal neurological crises.

VP is highly prevalent in South Africa and is estimated to affect between 10 and 20 thousand individuals (Day, 1986). This high prevalence is thought to be due to a founder effect with affected individuals descending from a 17th century Dutch immigrant (Dean, 1971; Meissner et al., 1996). Traditionally the disease has been diagnosed by the determination of porphyrin excretion patterns but such tests are of poor sensitivity and specificity. Identification of the major mutation in the South African VP population has led to the hope that DNA-based testing would reduce the need for these earlier tests (Hift et al., 1997).

One gene defect, a single base pair substitution (R59W) was identified by us and was shown to account for 96% of a sample of South African patients with VP (Meissner et al., 1996). In addition, we demonstrated the presence of another mutation (R168C) in a patient who was doubly heterozygous for this and for the R59W mutation, though we have not yet encountered heterozygous R168C substitution in any patient with biochemically expressed VP. Subsequently, an additional mutation (H20P) (Warnich et al., 1996; Hift et al., 1997) was reported in a further South African family. Thus there appeared to be an unusually high degree of genetic homogeneity for this condition in South Africa, which suggested that a diagnos-

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tic strategy based on the detection of the R59W mutation would be clinically useful.

We report here the identification of a further mutation in an unrelated South African family, some of whose members exhibit typical VP biochemistry and symptomatology. This is the first description of a deletion in the PPO gene in the South African VP community, provides further evidence for allelic heterogeneity in the South African VP population, and underlines the need for caution in recommending or relying on a single diagnostic test (determination of R59W) status in the diagnosis of this common disorder.

MATERIALS AND METHODS

Patients and Controls

The proband is a 31 year old female of mixed racial ancestry (European/Asian) who has the typical urine and stool porphyrin excretion profile of VP but tests negative for the three VP mutations described to date in South Africa. Clinical symptoms include cutaneous fragility, blistering and scarring in sun-exposed areas, and are in all respects typical of VP. She has never experienced an acute crisis. A year later, a female cousin also presented similar symptoms and was also diagnosed as having VP. Furthermore, 29 available family members were investigated by mutational analysis and with porphyrin biochemistry as described below. All but one of these were examined clinically. All gave informed consent for their participation prior to inclusion in the study . The study was formally approved by the Research Ethics Committee of the University of Cape Town Medical School (reference 051/98).

Biochemical Analysis

Quantitative thin-layer chromatographic fluoroscanning of stool, urine, and plasma was performed (Day, 1986; Day et al., 1978). A diagnosis of VP required the demonstration of characteristic porphyrin excretory patterns, including a stool protoporphyrin concentration > 200 nmol/g dry weight and coproporphyrin concentration > 50 nmol/g dry weight.

Mutational Analysis

Genomic DNA, isolated from whole blood (Parzer and Mannholter, 1991) was amplified by polymerase chain reaction (PCR) using exon specific primers. The primers utilised in the amplification of the 13 PPO exons were as follows: exon 3 as described by Meissner et al. (1996), exons 4, 5, and 7, 8, 9, 10, 11, 12/13 as described by Warnich et al. (1996), and exons 1, 2, and 6 as shown in Table 1.

A 317 bp fragment of exon 6 was generated. For exon 6 PCR was performed as follows: 95°C for 1 min; 35 cycles of (95°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec); 72°C for 7 min in an Omnigene thermal cycler (Hybaid).

For mutation detection, the PCR products of all 13 exons were screened using simultaneous single stranded conformational polymorphism (SSCP)-heteroduplex analysis (Spritz et al., 1992) on 1X MDE gels (FMC Bioproducts, Rockland, Maine USA) in 0.6% TBE. The amplified DNA product (5 μl) was mixed with an equal volume of loading buffer (95% formamide, 10 mM NaOH, 20 mM EDTA pH 8.0, 0.02% bromophenol blue, and 0.02% xylenecyanol). After denaturing at 95°C for 5 min followed by cooling on ice for 5 min, the sample was loaded immediately and run (300-400 V) overnight at room temperature in 0.6% TBE. The gels were run in both the presence and absence of 10% glycerol. In the absence of glycerol the voltage was halved. The exon (exon 6) which exhibited aberrant mobility shifts with SSCP and heteroduplex analysis was then sequenced. After purification of the product by QiaQuik DNA purification kit (Qiagen, Chatswell, CA) sequencing was performed on an ABI 373 (Applied Biosystems Incorporated, Foster City, California, USA) automated sequencer using dye terminator cycle sequencing. Sequencing was done in both directions using the relevant sense and antisense primers (DF6 and DR6).

As the 537delAT deletion creates an Mva1 (Boehringer Mannheim) cutting site, restriction analysis was performed on the exon 6 PCR prod-

TABLE 1. Primers Utilised in the Amplification of Exons 1, 2, and 6 of the PPO Gene

Exon	Primer	Sequence (5′–3′)	Product size (bp)
1	DF1	ATTCGGGGGGAGAACAGAGTGG	418
	DR1	CGATATGGACAGGCAGACCG	
2	DF2	TCTGCCTGTCCATATCGC	167
	DR2	ATTAAATGAAGCTCCCTC	
6	DF6	TATCCCACCCTCATTCCCTACCA	317
	DR6	ATTGAATAGCACCCCTTGTCC	

uct according to the manufacturers' instructions, with incubation for 3 h at 37°C and separation on a 1% agarose gel in TBE. Digestion produces two fragments of 158 and 157 bp in length.

RESULTS

Simultaneous SSCP-heteroduplex analysis of the PCR products of the 13 PPO exons revealed an aberrant SSCP mobility shift and the presence of a heteroduplex in exon 6 of both the proband and her cousin. This SSCP shift in the proband is shown in Figure 1. A heteroduplex is clearly visible in the proband lane but is absent in the control (Fig. 2). Figures 1 and 2 are upper (Fig.1) and lower sections (Fig. 2) of the same MDE gel. All exons were screened and no other abnormality was shown. Sequence analysis of exon 6 revealed a 2 bp deletion following nucleotide position 537 (537delAT; Genbank Accession No. AF075457), numbering from the A of the initiating methionine codon of the PPO cDNA sequence reported by Nishimura et al. (1995) (Fig. 3).

Mva1 restriction analysis was used to screen genomic DNA from 31 family members. Seventeen tested positive for the mutation. Thirteen of these 17 subjects showed biochemical evidence of VP in urine and stool porphyrin analysis whereas in another four, porphyrin excretion patterns were normal. These we class as "silent" cases (Kirsch et al., 1998). The mutation tracks with the disease, being present in all subjects who demonstrated

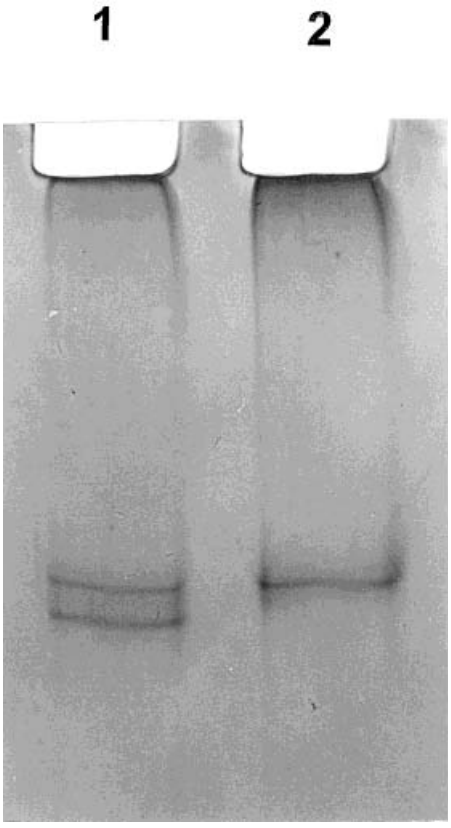

FIGURE 1. **SSCP of the PCR product of exon 6 on 1X MDE gel showing one band in the control individual (lane 2) and 2 bands in the proband (lane 1).**

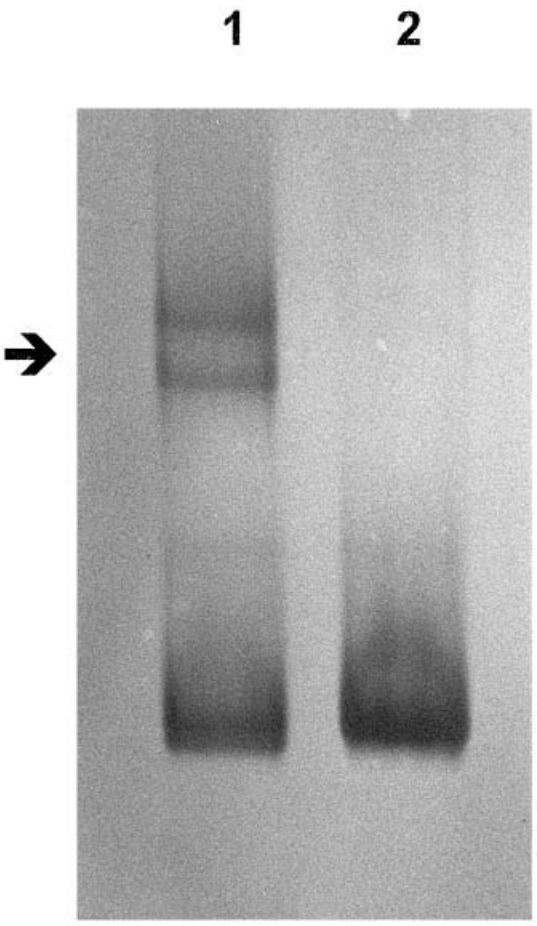

FIGURE 2. **Heteroduplex analysis of the PCR product on 1X MDE gel. Lane 1: proband; lane 2: control. The arrow indicates a heteroduplex.**

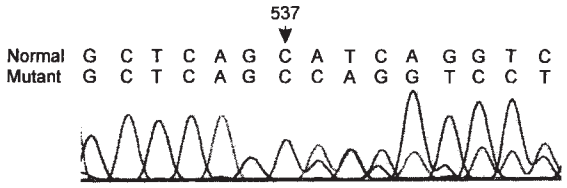

FIGURE 3. **Identification of a deletion in the PPO gene by DNA sequencing of the PCR product of exon 6 from the proband using primer DF6. It is apparent that the patient is heterozygous for the mutation as after the AT deletion both normal and aberrant sequence is apparent.**

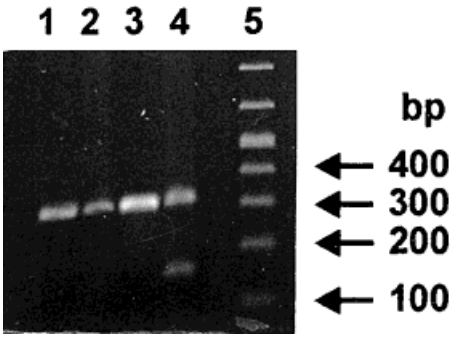

FIGURE 4. **Mva1 restriction analysis on 1% agarose. PCR amplified DNA of exon 6 from proband uncut (lane 3) cut (lane 4). Lanes 1 and 2 show DNA from control individual uncut and post digestion. Lane 5 is a DNA marker (Biomarker Low, BioVentures Inc., Murfreesboro, TN, USA).**

positive biochemistry. Sixteen of the 17 positive individuals were examined clinically and only three showed evidence of VP, the rest being asymptomatic. Forty-eight unrelated individuals of similar racial origin that were subjected to Mva1 restriction analysis as controls, were negative. Both restriction digestion (Fig. 4) and direct exon 6 sequencing of the proband (Fig. 3) and her cousin (data not shown) confirmed heterozygosity. Figure 5 depicts part of the pedigree which is extensive.

DISCUSSION

The 537delAT mutation results in a frameshift and produces a premature TGA stop codon 11 codons downstream. We propose that this may be responsible for the synthesis of a truncated protein with reduced or absented catalytic activity. Nonsense or frameshift mutations may result in

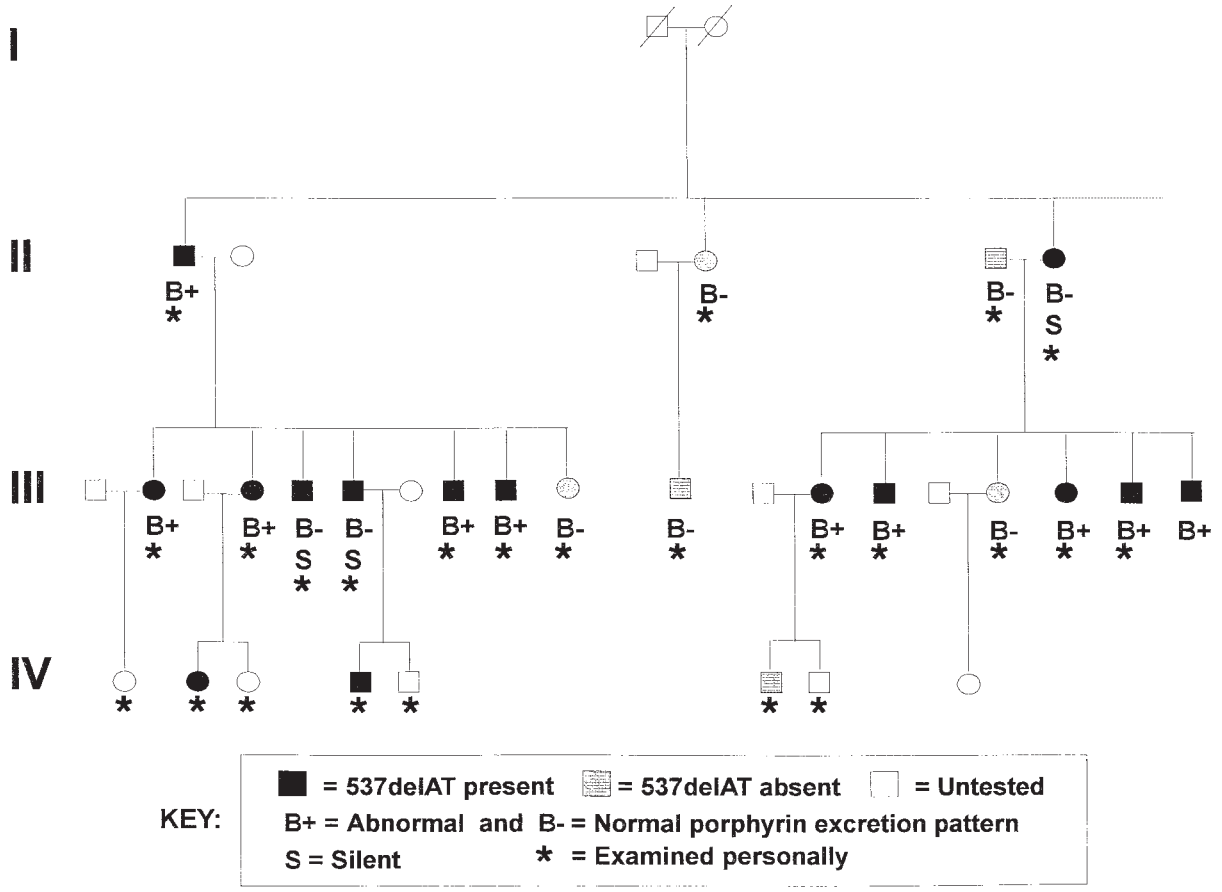

FIGURE 5. **Abridged pedigree illustrating four generations of this VP family. Mva1 restriction analysis was used to determine the presence (537delAT present) or absence (537delAT absent) of the 537delAT mutation. Individuals labelled B+ demonstrated a porphyrin excretion pattern consistent with VP; those labelled B- demonstrated a normal porphyrin excretion pattern. Silent (S) indicates those individuals who showed no clinical or biochemical evidence of the disease. All individuals in generation IV are well below pubertal age. Thus biochemical analyses were not performed because VP does not express biochemically, prepubertally.**

severe reductions in the steady state levels of cytoplasmic mRNA (Cooper, 1993). The mechanism by which a reduction in the mRNA levels may occur is unclear. One or several parameters may be affected e.g. transcription rate, efficiency of mRNA processing, transport to the cytoplasm, or mRNA stability (Cooper, 1993).The effects of the 537delAT mutation remain to be established and we are currently investigating mutant PPO mRNA levels as these may well be of importance in understanding the significance of this mutation. Construction of a site-directed mutant will provide further information.

Many different PPO mutations have been described from around the world in the short period since the first report, indicating significant allelic heterogeneity for this disease (de Rooij et al., 1997; Deybach et al., 1996; Frank et al., 1996a, 1996b; for review see Kirsch et al., 1998). Though the South African VP population is exceptional for the high degree of genetic homogeneity it demonstrates, our findings mandate caution in issuing a negative VP diagnosis based solely on the absence of the common R59W defect, because a minority of patients will harbour one of at least three mutations other than this. When DNA testing is employed in the screening of South African subjects for VP, 537delAT mutation testing by restriction digestion must be considered, in addition to testing for those mutations previously described.

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