Molecular basis of variegate porphyria: a missense mutation in the protoporphyrinogen oxidase gene

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Abstract

Variegate porphyria (VP) is an autosomal dominant disorder characterised by a partial defect in the activity of protoporphyrinogen oxidase (PPO), and has recently been genetically linked to the PPO gene on chromosome 1q22-23 (Z=6.62). In this study, we identified a mutation in the PPO gene in a patient with VP and two unaffected family members. The mutation consisted of a previously unreported T to C transition in exon 13 of the PPO gene, resulting in the substitution of a polar serine by a non-polar proline (S450P). This serine residue is evolutionarily highly conserved in man, mouse, and *Bacillus subtilis*, attesting to the importance of this residue. Interestingly, the gene for Gardner's syndrome (FAP) also segregates in this family, independently of the VP mutation. Gardner's syndrome or familial adenomatous polyposis (FAP) is also an autosomal dominantly inherited genodermatosis, and typically presents with colorectal cancer in early adult life secondary to extensive adenomatous polyps of the colon. The specific gene on chromosome 5 that is the site of the mutation in this disorder is known as APC (adenomatous polyposis coli), and the gene has been genetically linked to the region of 5q22.

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The porphyrias are disorders of porphyrin or porphyrin precursor metabolism resulting from inherited or acquired aberrations in one of the eight enzymes that control the porphyrin-haem biosynthetic pathway. Variegate porphyria (VP, OMIM 176200), one of the acute hepatic porphyrias, is characterised by a deficiency in the activity of protoporphyrinogen IX oxidase (PPOX, EC 1.3.3.4), the seventh enzyme in the pathway of haem biosynthesis that catalyses the conversion of protoporphyrinogen IX to protoporphyrin IX.¹

VP is an autosomal dominant disease displaying incomplete penetrance.^{2,3} PPO activity in affected subjects is decreased to approximately half normal levels in all tissues, although rare recessive cases with <20% PPO activity have been reported.^{4,5} The cutaneous symptoms that may be present in affected subjects are bullous photosensitivity, skin fragility with chronic scarring of areas exposed to the sun, and post-inflammatory hyperpigmentation.² The acute manifestations of VP include abdominal pain,

the passage of dark urine, and visceral and psychiatric symptoms arising from central, autonomic, and peripheral neuropathies.

Recently, the human PPO cDNA and gene were cloned and mapped to chromosome 1q22-23⁶⁻⁸ where we and others have identified pathogenetic mutations.⁹⁻¹⁵

In this study, we identified a mutation in a patient with VP and two clinically unaffected relatives. The mutation consisted of a T to C transition in exon 13 of the PPO gene that results in a previously unreported missense mutation, S450P. Our results establish this mutation as the fundamental lesion in a patient with the VP phenotype.

Interestingly, the family history also showed the occurrence of familial adenomatous polyposis (FAP) or Gardner's syndrome (OMIM 175100). FAP is an autosomal dominant disorder that typically presents with colorectal cancer in early adult life, following extensive adenomatous polyps of the colon, cutaneous epidermoid cysts, mesenchymal tumours on and under the skin, pigmented retinal lesions, jaw cysts, and osteomata.¹⁶ Polyps also develop in the upper gastrointestinal tract¹⁷ and malignancies may occur in other sites including the brain¹⁸ and thyroid.¹⁹ The specific gene on chromosome 5 that is the site of the mutation in this disorder is known as APC (adenomatous polyposis coli). Linkage studies resulted in mapping of the APC gene to the region of 5q22²⁰; in 1991 the gene was characterised and the first mutations were reported.^{21,22} In this family, the VP phenotype and FAP segregate independently, as some subjects with stigmata of FAP do not carry the VP mutation and vice versa. The proband with VP through whom this family was ascertained has only features of VP and no stigmata of FAP.

Materials and methods

CLINICAL MATERIAL AND BIOCHEMICAL ANALYSES
The proband is a 32 year old female of Italian, German, Scottish, Irish, and French ancestry who was diagnosed with variegate porphyria in 1995, on the basis of sun sensitivity and a porphyrin analysis. Her mother, three sibs, a nephew, and 50 unrelated control probands were also included in the study. Blood samples were collected from the proband, her mother, her three sibs, and 50 unrelated controls in tubes containing EDTA. A blood sample was not available from the proband's father. Because of his young age, cheekbrush samples were obtained from the nephew. All subjects provided informed consent for inclusion in the study, in accordance with guidelines set forth by the local institutional review board.

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Genomic DNA from all family members and the 50 control probands was isolated according to standard techniques.²³

Diagnosis of Gardner's syndrome was made by clinical examination of the skin, colonoscopy, and histopathological examination of colon biopsies.

PCR AMPLIFICATION AND MUTATION DETECTION

A mutation detection strategy was developed for individual PPO exons using PCR primers that were synthesised on the basis of the PPO cDNA and derived genomic sequence,^{6,8} owing to the small size of the introns. For amplification of exon 13 of PPO, the following primers were used: PPO exon 13L: 5'TCCCCAG-TATACACTAGGTC3', and PPO exon 13R: 5'GGAGCTCCAGCAATTTTAT3'.

PCR amplification resulted in a product 282 bp in size, containing 96 bp of intron 12, 183 bp of exon 13, and 3 bp of the untranslated region of the PPO gene. The numbers of the primers correspond to the published cDNA sequence, according to GenBank D38537,⁶ with the first nucleotide of the initiating methionine codon counted as No 1.

PCR was carried out on genomic DNA from all family members according to the following programme: 95°C for five minutes, followed by 40 cycles of 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, in an OmniGene thermal cycler (Marsh Scientific, Rochester, NY). For mutation detection, PCR products were subjected to CSGE analysis (conformation sensitive gel electrophoresis) as previously described in detail.²⁴ PCR products displaying a heteroduplex on CSGE analysis were purified using the high pure PCR product purification kit (Boehringer Mannheim) and sequenced using an ABI Prism 310 Genetic Analyzer from Applied Biosystems Inc. To verify the mutation, allele specific oligonucleotide (ASO) hybridisation was performed. In this method, PCR products are transferred by dot blotting onto a nylon membrane in duplicate. ASOs are designed so that one represents the wild type sequence, the other bears the mutation in the centre of the oligomer, and the T_m is approximately 60°C. In this case, the following oligonucleotides were used: oligo exon 13 wild type: 5'TGGCTGGAGCCTCCTATGA3', oligo exon 13 mutant: 5' GGCTGGAGCCCCCTATGA3'.

ASOs were end labelled using $\gamma^{32}\text{P}$ dATP and hybridised to the duplicate dot blots for one hour at 37°C. After washing at the T_m of the oligos for 10–30 minutes at high stringency ($0.1 \times \text{SSC}$, 0.5% SDS), the filters were exposed to autoradiography for visualisation of signals. There should be a signal of equal intensity in the lanes of a heterozygous subject using both the wild type and mutant oligos, with a stronger signal with the wild type oligo in a control and no signal with the mutant oligomer. If the person is homozygous for the polymorphism, there should be no wild type signal and only a signal with the mutant allele. To be convinced of the results of ASO analysis, both filters were stripped and rehybridised with

the opposite ASO to control for differences in sample loading.

Results

The proband (III.1 in fig 1A) suffered from increased sun sensitivity. Her mother, three sibs, and a nephew (II.1, III.2, III.3, III.5, and IV.1, respectively) were clinically unaffected with VP. Heteroduplex analysis of exon 13 of the PPO gene in this family showed a heteroduplex in the proband, one of her brothers, and the brother's son (III.1, III.3, and IV.1 in fig 1B, respectively). Automated sequence analysis of this PCR product in all family members showed the same sequence variant in the patient, the previously mentioned brother, and the brother's son (fig 1D). The mutation consisted of a T to C transition at nucleotide position 1348 of the PPO cDNA (numbered according to GenBank D38537, counting the first base of the initiating methionine as No 1). The base change results in a previously unreported missense mutation consisting of a serine to proline substitution at position 450 (S450P) in the deduced amino acid sequence. Heteroduplex analysis of exon 13 of the PPO gene was also performed in 50 unrelated controls and showed no heteroduplex formation indicative of the mutation (data not shown). To verify the sequence variation in exon 13, allele specific oligonucleotide (ASO) hybridisation was performed in all family members and two control probands. The proband, the brother, and his son (III.1, III.3, and IV.1 in fig 1C, respectively) showed a signal in the lanes of the wild type and the mutant oligos, indicating that they are heterozygous for the mutant allele.

With regard to Gardner's syndrome, three family members (I.3, I.4, and II.3 in fig 1A, respectively) had died from colon cancer. Colonoscopy in two other family members (III.7 and III.8) showed multiple adenomatous polyps in the colon. Two other family members (II.2 and III.3) showed cutaneous and skeletal stigmata of Gardner's syndrome but no atypical intestinal findings.

Discussion

In this study, we identified a missense mutation in the PPO gene in a patient with VP and two clinically unaffected family members by CSGE analysis, automated sequencing, and allele specific oligonucleotide (ASO) hybridisation. While the proband's asymptomatic father was not available for testing, we surmise that he is the carrier of S450P in the family, since it was not detected in the proband's mother, although the possibility of a paternal germline mosaic could not be excluded.

The mutation detected in our study consisted of a previously unreported T to C transition in exon 13 of the PPO gene, resulting in the substitution of a serine by a proline residue (S450P). Three lines of evidence suggest that this base substitution is responsible for VP in the clinically affected proband in the family studied. First, it causes an amino acid change from a polar serine residue to a non-polar proline residue at position 450 (S450P) in the

Figure 1 Mutation analysis in the VP patient in this study and her family members. (A) Pedigree of the nuclear family with regard to the occurrence of variegate porphyria (VP) and familial adenomatous polyposis (FAP): the proband suffering from VP (III.1) is indicated by a red, half filled circle. The three other family members carrying the S450P mutation are indicated by a red, half filled square (II.2, III.3, and IV.1, respectively). Two of them (II.2 and III.3, respectively) also show cutaneous and skeletal findings of FAP, indicated by an additional blue square within the symbol. The presence of FAP with colorectal findings is indicated by blue, half filled squares (I.3, I.4, II.3, III.7, and III.8, respectively). (B) Heteroduplex analysis of the family and two control probands (C1 and C2): heteroduplex formation in the proband (III.1), her brother (III.3), and her nephew (IV.1) in exon 13 of the PPO gene. (C) ASO hybridisation results: in exon 13, the proband (III.1), her brother (III.3), and her nephew (IV.1) show the mutant S450P allele (lower panel) and the wild type allele, whereas the other family members (II.1, III.2, and III.4) and the two control probands (C1 and C2) only show the wild type allele (upper panel). (D) Automated sequence of the forward strand of the PCR fragment of exon 13: T to C transition in exon 13 of the proband (lower panel, indicated by an arrow) compared with the wild type sequence of control proband C1 (upper panel).

PPO gene. This mutation may cause destabilisation of specific interactions in PPO and impair proper functioning of the encoded protein. Second, a comparison of nucleotide deduced amino acid sequences (fig 2) showed this serine residue to be strictly conserved in human,⁶ mouse over 90 million evolutionary years,²⁵ and over 500 million evolutionary years in *Bacillus subtilis*²⁶ through evolution, although their entire amino acid sequence identity is less than 20%. Therefore, this serine residue is believed to be of importance for proper functioning of the protein. Third, no other deviation in the proband's cDNA sequence of the PPO gene was detected.

hPPO	E S A R Q F L T A H R L P L T L A G A	Y E G V A V N D C I
mPPO	D S A M Q F L T A Q R L P L T L A G A	Y E G V A V N D C I
hemY	K E L R E A L A S A Y P G V Y M T G A	F E G V G I P D C I
S450P		

Figure 2 Alignment of deduced amino acid sequences from codon 431 to 460 of human PPO (hPPO), mouse PPO (mPPO), and the hemY gene product from *Bacillus subtilis* (hemY). The identical residues in codon 450 and the substitution derived from the described missense mutation are shaded and bold.

The results presented here show that the clinical expression of defects in the PPO gene in families with VP is highly variable and underline the incomplete penetrance of the disease. The reasons for the different patterns of phenotypic expression in this heterogeneous disorder are not well understood yet. In our study, one of the patient's sibs and his son carried the S450P mutation, but were asymptomatic for VP. Interestingly, the proband's brother who carries the mutation S450P also shows sebaceous cysts and osteomata characteristic of Gardner's syndrome. Colonoscopy in all offspring of II.2 was negative for polyps. Biochemically or clinically "silent" carriers of gene defects are often observed in families with other types of porphyrias, such as erythropoietic protoporphyria, acute intermittent porphyria, or hereditary coproporphyria.²⁷ These silent cases can become manifest after exposure to endogenous or exogenous factors, such as ingestion of porphyrinogenic drugs, hormonal fluctuation, or exposure to UV light. Carbohydrate restriction is also known to provoke acute episodes of VP.²⁸ Sometimes, however, exposure to known inducers may fail

to elicit either abnormal clinical or biochemical signs, as in the exposure to oestrogen in some cases of familial porphyria cutanea tarda.²⁷

Our study shows the variability in clinical expression of VP in a family with missense mutation in the PPO gene and the clinical features of Gardner's syndrome. Further investigations are under way to elucidate the molecular basis of VP, establish genotype to phenotype correlations by the expression of mutant alleles, and understand additional and unusual mechanisms of inheritance of the disease.

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