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Molecular analysis of the *UROD* gene in 17 Argentinean patients with familial porphyria cutanea tarda: Characterization of four novel mutations

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ABSTRACT

Porphyria cutanea tarda (PCT) is caused by decreased activity of uroporphyrinogen decarboxylase (UROD) in the liver. The disease usually occurs in adulthood and is characterized by cutaneous photosensitivity, hyperpigmentation, skin fragility and hypertrichosis, due to the accumulation of porphyrins produced by oxidation of uroporphyrinogen and other highly carboxylated porphyrinogens overproduced as a result of the enzyme deficiency. PCT is generally sporadic, but about 20–30% of patients have familial-PCT (F-PCT) which is associated with heterozygosity of mutations in the *UROD* gene. In the present study we have found the molecular defect in seventeen unrelated Argentinean patients with F-PCT, identifying a total of eleven *UROD* gene mutations: four novel and seven previously described. The novel mutations were: a guanine insertion at the 5′ splice junction of intron 2, a three nucleotide deletion causing the lost of valine 90, a deletion of 22 bp in exon 6 and a deletion of part of the polyadenylation signal. Prokaryotic expression studies showed that the novel amino acid deletion resulted in an inactive protein. Mutations c.10insA and p.M165R, previously found in Argentinean patients, were recurrent in this study; they are the most frequent in Argentina accounting for 40% of the mutant alleles characterized to date.

1. Introduction

Porphyria cutanea tarda (PCT; OMIM 176100), the most common type of porphyria is due to a decreased activity of uroporphyrinogen decarboxylase (UROD; E.C.4.1.1.37). This cytosolic enzyme catalyzes the fifth step in the heme biosynthetic pathway (Fig. 1), the sequential decarboxylation of the four acetate side chains of uroporphyrinogen to form coproporphyrinogen [1,2]. PCT usually develops in adulthood and is characterized by hepatic accumulation of uroporphyrinogen and other highly carboxylated porphyrinogens that circulate in plasma and are excreted and found as porphyrins in urine [2,3]. The overproduction of porphyrins is the reason for the major clinical manifestations of the disease, cutaneous photosensitivity with blistering on areas exposed to sun, skin fragility, hyperpigmentation, and hypertrichosis [2]. Three clinically similar forms of PCT can be distinguished: sporadic (S-PCT or type I), familial (F-PCT or type II) and type III PCT [2,4,5]. F-PCT is an autosomal dominant disorder with low penetrance in which the UROD activity is reduced to about 50% in all tissues due to mutations in heterozygosis in the *UROD* gene. F-PCT represents about 20–30% of PCT patients, who are predisposed to develop the disease. Most PCT patients have S-PCT, which is not associated with mutations in the *UROD* gene and the deficiency in enzymatic activity is restricted to the liver [2,4,5]. Type III PCT is a rare form similar to S-PCT but, while the S-PCT does not show a familial pattern, type III PCT is associated with a familial history for the disease [5]. The clinical manifestation of PCT is frequently associated with exposure to precipitating factors including iron overload, alcohol abuse, use of estrogens, and hepatitis C virus infection [2,3,6–8]. Hereditary hemochromatosis associated with the *HFE* gene is the most common genetic cause of iron overload and inheritance of *HFE* mutations is prevalent in PCT patients [7]. The clinical expression of the disease is a consequence of a marked deficiency in the UROD activity in the liver caused by a competitive inhibitor (uroporphomethene) generated in an iron-dependent oxidation of uroporhyrinogen [9]. *UROD* mutations in homozygosis or compound heterozygosis are a rare condition known as hepatoerythropoietic porphyria (HEP) with more severe clinical and biochemical features than F-PCT, characterized by the onset in early childhood and a phenotype similar to that of congenital erythropoietic porphyria [2].

Abbreviations: UROD, uroporphyrinogen decarboxylase; PCT, porphyria cutanea tarda; F-PCT, familial porphyria cutanea tarda; S-PCT, sporadic porphyria cutanea tarda; HEP, hepatoerythropoietic porphyria; HFE, hemochromatosis gene; RBC, red blood cells; PPI, plasma porphyrins index; PTC, premature termination codon; NMD, nonsense-mediated mRNA decay; 3′UTR, 3′ untranslated region.

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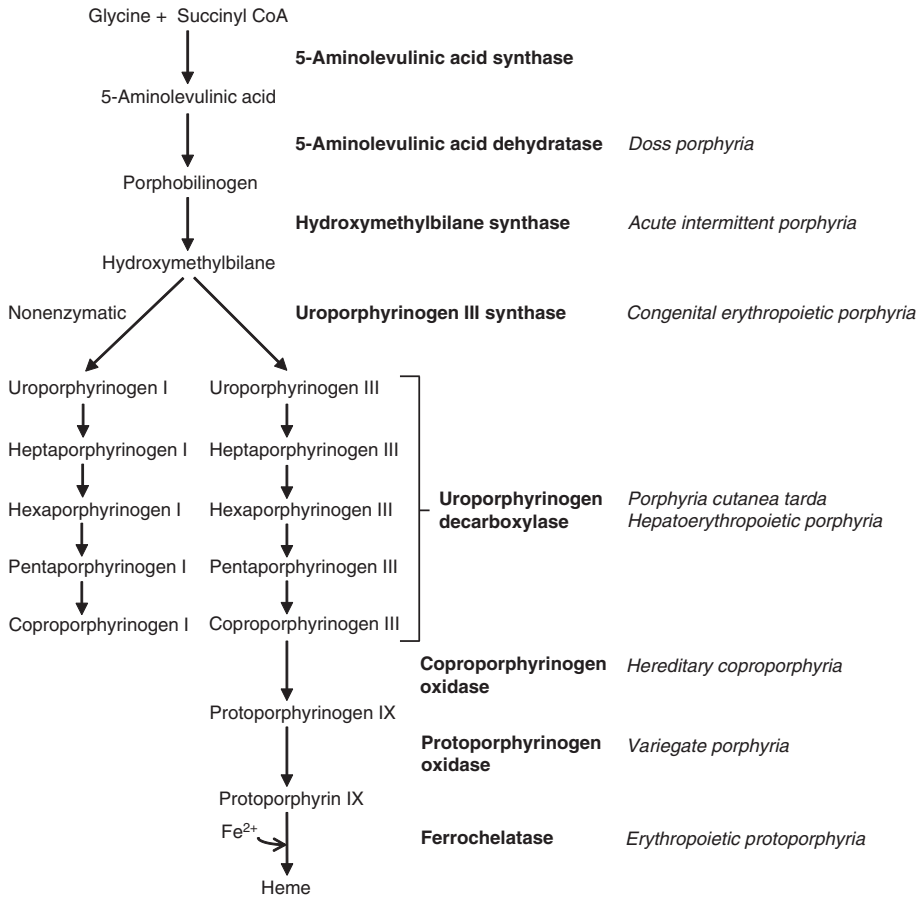

Fig. 1. Heme biosynthetic pathway and the associated porphyrias.

The human *UROD* gene has been mapped to chromosomal region 1p34 [10], spans a genomic interval of 3.6 kb, contains a single promoter, 10 exons, and a polyadenylation signal with a canonical AATAAA element [11,12]. The mRNA has 1.2 kb and encodes a 367 amino acid polypeptide with a molecular weight of approximately 41 kDa [13]. The human UROD protein is a homodimer that belongs to the $(\alpha/\beta)_8$ barrel family [14]. To date, 108 mutations in the *UROD* gene have been identified in patients with F-PCT and/or HEP (Human Gene Mutation Database (HGMD); http://www.hgmd.cf.ac.uk/ac/index.php).

Twelve mutations in the *UROD* gene were previously identified in seventeen Argentinean probands with F-PCT or HEP [15–17]. In the present study, we found the molecular defect of the *UROD* gene in seventeen unrelated Argentinean probands with F-PCT, identifying eleven distinct mutations including four novel ones.

2. Material and methods

2.1. Patients and biochemical determinations

Seventeen unrelated F-PCT patients, 11 males and 6 females from different geographical regions of Argentina were included in this study. They were referred to the CIPYP from several health centres with presumed PCT diagnosis. In eight patients, aged between 5 and 14, the onset of clinical symptoms occurred in childhood, while in nine patients it was during adulthood, aged between 23 and 64.

The urinary porphyrins, their excretion pattern, the plasma porphyrin index (PPI) and the erythrocytic UROD activity were determined in accordance with the methodologies described [18–20]. PCT diagnosis was based on typical cutaneous lesions, elevated amounts of porphyrins in plasma (porphyrin peak at 618 nm), as well as an increase in the urinary excretion of porphyrins with predominance of uroporphyrin and heptaporphyrin. The F-PCT diagnosis was based on a reduced erythrocytic UROD activity about 50% of normal value.

All patients gave informed consent prior to their inclusion in the study. The work was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethical Committee of the Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP, Hospital de Clínicas, CONICET-UBA).

2.2. Mutation analysis of the UROD gene

Blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA), and genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. The entire *UROD* gene was amplified in five overlapping PCR fragments using the primer pairs and the conditions previously described [16]. PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK), and sequenced with the PCR primers using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Sequencing reactions were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) and analyzed on an ABI 3130 xl Genetic Analyzer (Applied Biosystems). All mutations were confirmed by sequencing both strands of at least two different PCR products. Nucleotides were numbered according to the cDNA sequence derived from the *UROD* genomic sequence (GenBank accession number: AF047383, [15]), in which the A of the ATG translation-initiation codon is numbered as +1.

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2.3. In silico analysis

The possible consequences of the newly identified splice site insertion on mRNA splicing were assessed *in silico* analyzing the genomic sequence using the following prediction programs: Human Splice Finder (http://www.umd.be/HSF) [21], Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) [22] and MaxEntScan (http://www.genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) [23]. The latter was used to estimate the splice site strength of predicted sites by the first programs. In the MaxEntScan, scores of the 9-nucleotides sequences of donor sites were calculated selecting for the analysis, the Maximum Entropy Model (MAXENT), Maximum Dependence Decomposition Model (MDD), First-order Markov Model (MM) and Weight Matrix Model (WMM).

2.4. Prokaryotic expression of p.V90 deletion

The normal and V90-deleted *UROD* alleles were expressed in *Escherichia coli* strain JM109 (Promega. Madison, WI, USA) using the expression vector pKK223-3 (Pharmacia Biotech Inc., Piscataway, NJ, USA). A fragment of *UROD* cDNA carrying the c.267_269 deletion and restriction sites for cloning, was generated by PCR-based site-directed mutagenesis, using as a template the normal expression plasmid (pKK-UROD-wt), in which the wild-type human *UROD* cDNA was cloned into the *EcoRI*/*HindIII* sites of the pKK223-3 [15,24,25]. The PCR reaction was performed with the sense primer 5′-CCGGAATTCATGGAAGCGAATGGG-3′ containing a restriction site for *EcoRI* (underlined nucleotides), and the antisense primer 5′-GCCAGGTACCATGGTCACCTCCATGCCCAGTGCCTGGGGTACAAGGATG-TCGGA-3′ containing a restriction site for *KpnI* (underlined nucleotides) and the three nucleotides deletion. The PCR amplification was carried out in a final volume of 100 μl, containing 10 ng of plasmid, 1 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 30 pmol of each primer and 2 units of Taq DNA polymerase (Biotools, B & M Labs, Madrid, Spain), in 1× reaction buffer (supplied by Biotools). PCR conditions were: an initial denaturation step at 95 °C (3 min), followed by 30 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (30 s). The PCR product was digested with the restriction endonucleases *EcoRI* and *KpnI* (MBI Fermentas, Quimigen, Spain) and purified by the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK). The purified fragment was ligated using T4 DNA Ligase (Roche, Mannheim, Germany) into the *EcoRI*/*KpnI* sites of pKK-UROD-wt and the resulting plasmid was transformed into *E. coli* JM109, thus generating the mutant construct pKK-UROD-V90del. The integrity of the expression construct was checked by automated sequencing in an ABI 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems).

Bacterial clones containing one of the plasmids pKK223-3, pKK-UROD-wt or pKK-UROD-V90del were grown to log phase and induced with 5 mM isopropylthiogalactoside (MBI Fermentas) for 3 h. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline. Cell pellets were resuspended in lysis buffer (250 mM potassium phosphate, pH 6.0, 0.1% Triton-X 100) and disrupted by sonication. The bacterial lysates were centrifuged and the UROD activity was measured in the supernatants using pentaporphyrinogen I (Porphyrin Products Inc., Logan, UT, USA) as substrate, as previously published [26]. The porphyrins produced were analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection, in a Shimadzu apparatus (Shimadzu, Kyoto, Japan), according to the method of Lim et al. [27]. The specific activity was calculated as nmol of coproporphyrinogen I formed per hour per mg protein. Residual activity was determined from the mean specific activities (SA) of four independent experiments by dividing $100\times(SA_{[pKK\text{-}UROD\text{-}V90del]} - SA_{[pKK223\text{-}3]})$ by $(SA_{[pKK\text{-}UROD\text{-}wt]} - SA_{[pKK223\text{-}3]})$.

3. Results

The biochemical profile and genotype of the F-PCT patients studied are shown in Table 1. In these probands, four novel and seven already reported mutations were identified at heterozygous state. The novel mutations were one insertion and three deletions (Table 1, Supplementary Fig. 1). The first is a single guanine insertion at the 5′ exon–intron junction of intron 2, designated as IVS2 + 1insG. This insertion disrupts the wild-type donor splice junction CAGgtaaga by changing the position of the invariant "gt" dinucleotide, generating a potential exon/intron junction AGGgtaaga with an AGG defining the end of exon 2. The *in silico* analysis using the Human splice Finder indicated for the authentic wild-type donor junction (CAGgtaaga) a high consensus value (CV) of 97.66 (in a scale of 0–100), being therefore a strong donor site (CV > 80, [21]). Moreover no other strong site was predicted in the vicinity of this site. The same analyses on the mutated sequence recognize the novel AGGgtaaga nonamer as a strong donor junction with a lower CV of 91.85. Concordant results were obtained using the Splice Site Prediction by Neural Network and the MaxEntScan models (MAXENT, MDD, MM, WMM), with scores for the novel site vs. the authentic wild-type site of: 0.99 vs. 1.00 by the Neural Network program and 9.21, 12.98, 8.36, 9.08 vs. 10.77, 14.98, 11.11, 11.73 by the respective MaxEntScan models.

One of the three novel deletions was a 3 bp deletion in exon 4, designated as c.267_269delTGT, which was identified in two probands. This mutation involves the last base of codon 89 (GTT) and the two first bases of codon 90 (GTA), both codifying valine, therefore resulting in a valine codon (GTA) and in the deletion of the second valine (p.V90del). The other novel deletion was a 22 bp deletion in exon 6 that causes a frameshift generating a premature translation termination signal, 6 codons downstream, designated c.573_594del22. The last new mutation was the deletion of two adenines at the 3′ UTR of the gene that removes part of the polyadenylation signal, designated c.1104 + 62_63delAA.

The remaining seven mutations found in these patients, as already stated, were previously described (Table 1). In the present work, the p.D79N and p.H220P mutations were found for the first time in Argentina, while the c.10insA and p.M165R were found in more than one proband.

The novel amino acid deletion (p.V90del) expressed in *E. coli* rendered a residual activity less than 1% of the wild-type UROD activity.

4. Discussion

In this study, four novel *UROD* gene rearrangements were identified. The single guanine insertion IVS2 + 1insG occurred at side or between two guanines: the last nucleotide of exon 2 and the first nucleotide of intron 2 (Supplementary Fig. 1), disrupting the donor junction of this intron [31]. Unfortunately, no sample was available from this patient to study the effect of the mutation on mRNA splicing by Reverse Transcription-PCR analysis. However, the additional guanine at this position modifies the donor site generating a new sequence suitable to serve as 5′ splice junction, one nucleotide downstream of the natural position, from CAGgtaaga to AGGgtaaga (consensus sequence: (A/C)AGgt(a/g)agt). The *in silico* analysis using three different web-based prediction tools suggested that this novel junction most probably serves as a donor splice site of intron 2. This splicing would leave an additional guanine at the end of exon 2, causing a frameshift with the introduction of a premature termination codon (PTC) four nucleotides downstream. Therefore, the degradation of the mutant transcript by nonsense-mediated mRNA decay (NMD) is the most probable to occur [32,33]. One of the two novel exonic deletions, c.573_594del22, also causes a frameshift generating a PTC, therefore the mutated transcript is the most likely to be subject to NMD. The other exonic deletion, c.267_269delTGT, predicts the lost of valine 90 but maintains the downstream reading frame.

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Table 1
Biochemical profile and genotype of the patients from this study.

Patient (sex)	Onset (years)	Urinary porphyrins (μg/24 h)	PPI	UROD activity[a]	Mutation[b]	Location	Reference
P1 (F)	23	6511	5.8	50.4	c.10insA	Exon 1	[15]
P2 (F)	6	1424	2.8	47.4			
P3 (F)	7	1298	7.8	48.6			
P4 (M)	5	14 334	9.0	46.5			
P5 (M)	52	1209	1.9	54.4	IVS2 + 1insG	Intron 2	This study
P6 (M)	64	6364	3.3	34.0	c.235 G > A; p.D79N	Exon 4	[28]
P7 (M)	44	432	1.7	55.6	c.239 C > G; p.A80G	Exon 4	[29]
P8 (M)	12	6168	3.0	54.7	c.267_269delTGT	Exon 4	This study
P9 (M)	5	2412	3.0	49.8			
P10 (M)	29	1051	2.2	48.7	c.573_594del22	Exon 6	This study
P11 (F)	44	4152	4.6	53.0	c.494 T > G; p.M165R	Exon 6	[15]
P12 (M)	36	7884	6.3	55.2			
P13 (F)	5	2295	2.4	51.4			
P14 (F)	27	1030	2.2	39.1	c.659A > C; p.H220P	Exon 7	[30]
P15(M)	13	1526	1.9	57.6	c.912 C > A; p.N304K	Exon 9	[15]
P16 (M)	14	4944	3.9	62.6	IVS9-1 G > C	Intron 9	[16]
P17 (M)	34	2075	7.8	35.6	c.1104 + 62_63delAA	3′UTR	This study

Values of porphyrins in urine and PPI were those determined at diagnosis.
Normal values: Urinary porphyrins: up to 250 μg/24 h; PPI: up to 1.3 (at λ: 618 nm); UROD activity: mean ± SD: 4.2 ± 0.6 nmol coproporphyrinogen III/h/ml RBC.
[a] UROD: Erythrocytic uroporphyrinogen decarboxylase activity is expressed as a percentage of normal value.
[b] Absence of these sequence deviations was confirmed in 50 unrelated healthy (non-porphyric) Argentinean individuals. Reference sequence: GenBank Accession number, AF047383.

Prokaryotic expression studies demonstrated that an allele carrying this deletion encodes an inactive protein. Valine 90 is located within the HD-helix motif of UROD (formed by amino acids 89 to 93), near important residues for functioning of the enzyme [14,34]. The deletion of this hydrophobic and bulk aminoacid could cause a significant structural alteration which would explain the lost of activity. Finally, a deletion of two adenines was identified in the 3′UTR of *UROD* gene (c.1104 + 62_63delAA). This deletion occurred in a track of four adenines in the 3′ regulatory region, altering the last nucleotide of the polyadenylation signal element AATAAA that was changed into an AATAAG hexamer (Supplementary Fig. 1). In this case, the mutation would most likely affect the strength of this signal, causing impaired 3′ end formation and the instability of the mRNA transcribed from the mutant allele. This result was often found for mutations altering this motif (consensus sequence: A(A/T)TAAA) described in other genes [35]. Indeed, mutations leading a guanine at the last position of this signal were found to be deleterious in other genes causing diseases such as thalassaemias [35]. Moreover, previous studies on the effect of different mutations in the AATAAA polyadenylation signal showed that a transcript containing the AAUAAG hexamer exhibited a markedly reduced polyadenylation efficiency *in vitro* and was lowly expressed in different mammalian cell lines [36,37]. To date, this is the third mutation found in the 3′UTR of the *UROD* gene and the first mutation in the highly conserved AATAAA signal. The mutations previously identified in the 3′UTR of the *UROD* were two single nucleotide substitutions, three and seventy base-pairs after the stop codon, designated c.1104 + 3 G > A and c.1104 + 70 G > A, respectively [38–40].

In addition, seven known mutations were identified, two of them (p.D79N and p.H220P) were found for the first time in Argentina. The mutation p.H220P has already been described in a compound heterozygous HEP case p.Q134V/p.H220P [30], whereas the mutation p.D79N was reported in a Spanish patient with F-PCT [28]. Two mutations, c.10insA and p.M165R, were recurrent in our study and were identified in four and three probands, respectively. The c.10insA mutation is the most prevalent *UROD* mutation in Argentina and has not yet been reported in other countries to date [15,16]. The p.M165R mutation was described in an Argentinean F-PCT proband and later in patients with F-PCT from USA and Italy [15,41,42].

In the Argentinean population, the frequency of F-PCT was estimated to be 25% of PCT cases [20]. On the basis of the present study and three previous reports [15–17], 18 different mutations were identified in 33 F-PCT and in one heteroallelic HEP Argentinean probands. At present, roughly half of these mutations have not been reported in other populations. Thirty-five mutant alleles from the Argentinean patients were characterized, the most frequent mutations were c.10insA and p.M165R, found in 10 (28.6%) and 4 (11.4%) alleles, respectively. No genotype–phenotype correlation between the different *UROD* mutations and the severity of PCT was observed in the patients of the present study and the previous reports [15,16], suggesting participation of other factors in these individuals. In PCT, multiple risk factors, genetic and environmental, contribute to its manifestation [2].

In summary, eleven *UROD* mutations were identified in seventeen Argentinean F-PCT probands, four of them were novels. One of these novel mutations is the first mutation described at the polyadenylation signal element AATAAA of the *UROD* gene. The novel mutations were most likely the cause of reduced UROD activity observed in the affected probands and then, they can predispose to PCT development. These data will allow the precise identification of asymptomatic carriers in the families of the patients studied, to provide counselling to those individuals regarding avoidance of disease-precipitating factors. The overall results of this population indicate that F-PCT in Argentina is molecularly heterogeneous. However two mutations represent together 40% of the mutant alleles characterized to date, one of them (c.10insA) being found approximately in one third of the patients. Moreover, the identification of four novel mutations adds to the molecular heterogeneity underlying the F-PCT worldwide.

Conflict of interest statement

The authors declare no conflict of interest.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2012.02.002.

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