The molecular basis of porphyria cutanea tarda in Chile: Identification and functional characterization of mutations in the uroporphyrinogen decarboxylase gene

Poblete-Gutiérrez P, Mendez M, Wiederholt T, Merk HF, Fontanellas A, Wolff C, Frank J. The molecular basis of porphyria cutanea tarda in Chile: Identification and functional characterization of mutations in the uroporphyrinogen decarboxylase gene.

Abstract: The porphyrias are heterogeneous disorders arising from predominantly inherited catalytic deficiencies of specific enzymes in heme biosynthesis. Porphyria cutanea tarda (PCT) results from a decreased activity of uroporphyrinogen decarboxylase, the fifth enzyme in heme biosynthesis. The disorder represents the only porphyria that is not exclusively inherited monogenetically. In PCT, at least two different types can be distinguished: acquired/sporadic (type I) PCT, in which the enzymatic deficiency is limited to the liver and inherited/familial (type II) PCT, which is inherited as an autosomal dominant trait with a decrease of enzymatic activity in all tissues. In an effort to characterize the molecular basis of PCT in Chile, we identified eight mutations in 18 previously unclassified PCT families by polymerase chain reaction, heteroduplex analysis, and automated sequencing. To study the role of these mutations in disease causality, *in vitro* expression of all novel missense mutations was studied. Our results indicate that the frequency of familial PCT in Chile is approximately 50%, thus, to our knowledge, representing the highest incidence of familial PCT reported to date. The data further emphasize the molecular heterogeneity in type II PCT and demonstrate the advantages of molecular genetic techniques as a diagnostic tool and in the detection of clinically asymptomatic mutation carriers.

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Introduction

The porphyrias are rare metabolic diseases, which arise from an either inherited or acquired dysfunction of specific enzymes involved in heme biosynthesis. Dysfunction of seven of these enzymes leads to an accumulation of the preceding metabolites with a measurable increase of porphyrins and/or porphyrin precursors.

Porphyria cutanea tarda (PCT) (OMIM 176100) is the most frequent type of porphyria throughout the world and results from a decreased catalytic activity of uroporphyrinogen decarboxylase (URO-D), the fifth enzyme in heme biosynthesis. The enzyme is localized in the cytosol and catalyzes the sequential oxidative decarboxylation of the four acetic acid side chains of uroporphyrinogen to form the tetracarboxylic coproporphyrinogen (1,2).

According to the major site of expression of URO-D, at least two types of PCT can be distinguished: a sporadic (acquired) variant, designated type I PCT, in which the enzymatic deficiency is exclusively expressed in the liver and a familial

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(hereditary) variant, designated type II PCT, in which the catalytic enzymatic defect is detected in all tissues (2,3). Currently, the ratio between type I and type II PCT is estimated to be approximately 3 : 1 to 4 : 1 (1,2,4).

Noteworthy, however, not every PCT patient with a positive family history will necessarily be suffering from type II PCT. Recently, Elder (5) reported several families in which more than one individual was unequivocally affected with PCT. While these individuals revealed the typical clinical and biochemical characteristics of overt disease, normal URO-D activities were measured in red blood cells. This latter variant of the disease has been designated type III PCT and, in sum, there is increasing evidence that some facets of the etiology of PCT are not completely elucidated yet (5).

The diagnosis of PCT is made on the basis of cutaneous manifestations, a characteristic urinary porphyrin excretion profile, and, in some laboratories, by measuring URO-D activities in red blood cells. The skin findings include increased photosensitivity due to photosensitization by porphyrins and skin fragility as well as blistering, erosions, crusts, and milia on the sun-exposed areas of the body. Additionally, hyperpigmentation, hypertrichosis, sclerodermoid plaques, and scarring alopecia can be observed. Biochemically, an increased excretion of uroporphyrin (type I isomers > type III isomers), 7-carboxyl porphyrins (type III isomers > type I isomers), and coproporphyrin in the urine can be found. Enzymatically, URO-D activity is decreased by approximately 50% in red blood cells of individuals suffering from type II PCT.

The clinical manifestation of PCT is usually precipitated by different triggering factors, among them alcohol, estrogens, polychlorinated hydrocarbons, hemodialysis in patients with renal failure, iron, and viral infections such as hepatitis C and human immunodeficiency virus (HIV). Interestingly, patients suffering from PCT have a higher risk of developing hepatocellular carcinoma and therefore should be examined in regular intervals for the occurrence of this tumor (2,3,6).

Type II PCT is usually inherited as an autosomal dominant trait, displaying incomplete penetrance, as not all individuals carrying a mutation in the *URO-D* gene will develop the clinical phenotype (7). However, rare cases in which the genetic defect was inherited in a recessive fashion have also been described, and these patients are referred to as suffering from hepatoerythropoietic porphyria (8–14).

In heterozygotes suffering from type II PCT, URO-D activity is decreased by approximately 50% in all cells. The human URO-D cDNA and gene were previously cloned and mapped to chromosome 1p34 (15–17), and different groups have reported mutations in the URO-D gene in families with type II PCT (18–27).

Here, we studied 18 unrelated and previously unclassified PCT patients of Chilean origin by polymerase chain reaction (PCR)-based techniques. We identified six different mutations in the *URO-D* gene in eight unrelated patients. The mutations consisted of five missense mutations and one frameshift mutation. Whereas three of these mutations are novel ones, the other three have been reported previously by other groups (8,14,20). The likelihood of the novel missense mutations in causing disease was demonstrated by *in vitro* expression studies.

Our data comprise the first genetic studies of PCT in Chile. Interestingly, the results of our molecular analyses revealed that the ratio between familial and sporadic cases is approximately 1 : 1. Thus, the incidence rate of type II PCT in Chile appears to be higher than that reported from any other country in the world to date. Furthermore, our results emphasize the molecular heterogeneity in familial PCT and the utility of molecular genetic techniques in studying asymptomatic family members.

Materials and methods

Diagnosis, clinical material and DNA extraction

A diagnosis of PCT was established on the basis of cutaneous photosensitivity and typical skin symptoms on the sun-exposed areas like hands, forearms and face in combination with a characteristic porphyrin excretion profile in the urine consisting of increased values for urinary uroporphyrin (10 times higher than the upper normal range; type I isomers > type III isomers), 7-carboxyl porphyrins (type III isomers > type I isomers), and coproporphyrin. Urine levels of the porphyrin precursors δ-aminolevulinic acid and porphobilinogen were within normal ranges. Further, the concentration of isocoproporphyrin in the feces was measured by high-performance thin-layer chromatography.

Blood samples were collected from 18 unrelated and unclassified PCT patients and 100 control individuals of Chilean origin in tubes containing ethylenediaminetetraacetic acid. All individuals gave informed consent for inclusion in the investigation, in accordance with guidelines set forth by the local institutional review board. Genomic DNA was isolated according to standard techniques (28).

PCR amplification and mutation screening

A mutation-detection strategy for the *URO-D* gene was developed based on PCR amplification of all coding exons and the adjacent splice sites using primers that were designed for this study. All primer sequences and annealing conditions are described in Table 1. PCR for the amplification of the coding regions of the *URO-D* gene was carried out according to the following program: initial denaturation at 95°C for 5 min,

Table 1. Primer sequences, annealing conditions, and amplified regions of the uroporphyrinogen decarboxylase gene in this study

Primer	Sequence (5′→3′)	Annealing (°C)	Amplified region
PCT ex 1F	GGGCAGGCTCAGATTCAGGTTA	53	Exon 1
PCT ex 1R	TGGTTGGAAATCCTGAGGCATC		
PCT ex 2F	TGGAGGAGGTAGGATAGCGGTC	53	Exon 2
PCT ex 2R	ACACGTGCTGAAAAAGTCCTGG		
PCT ex 3/4F	AATCTAGATAAAACTCCGGAGG	55	Exons 3 and 4
PCT ex 3/4R	GCGTCCTTGGATTATATTCTAG		
PCT ex 5F	TGGATCGAGGGAAAAACTAAGG	55	Exon 5
PCT ex 5R	CTACCCCAGACCTTCCAGAGTG		
PCT ex 6F	ATCACTCTGGAAGGTCTGGGGT	53	Exon 6
PCT ex 6R	ACTCTTGCTTCTCGGCCTTACA		
PCT ex 7F	GAGGTGGATTTTGTATGTGG	53	Exon 7
PCT ex 7R	GCCTTGCTACAACCACTAAT		
PCT ex 8/9F	TGGAGGGCAGCAGAAGTACAGT	53	Exons 8 and 9
PCT ex 8/9R	ATACAACCACAGGCCACAGGAG		
PCT ex 10F	TTGTGTTTATGCTTCATGCCTG	53	Exon 10
PCT ex 10R	TCCGAAACTTTTATTGTCCTGG		

followed by 35 cycles of denaturation at 95°C for 45 s, annealing at primer-specific temperatures as summarized in Table 1 for 1 min, and extension at 72°C for 1 min and 15 s, followed by a final extension at 72°C for 10 min, in a Biometra® TGradient thermal cycler (Whatman Biometra®, Göttingen, Germany). Each amplification reaction contained approximately 100 ng of genomic DNA, 50 ng/μl of each forward and reverse primer and 35 μl of Platinum Taq® PCR Super Mix (Invitrogen® Life Technologies, Karlsruhe, Germany) in a total volume of 38 μl.

For mutation screening, PCR products were subjected to conformation-sensitive gel electrophoresis (CSGE) analysis, as previously described (29). PCR products displaying a heteroduplex on CSGE analysis were purified in a first step, using the High Pure PCR product purification kit (Boehringer Mannheim, Indianapolis, IN, USA). In a second step, PCR fragments were purified on Edge Centriflex columns (Edge BioSystems, Gaithersburg, MD, USA) and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). The PCR products of patients who did not reveal any heteroduplex formation upon screening were directly sequenced to assure that no mutation was missed, even if not detectable by CSGE analysis.

Prokaryotic expression of missense mutations P150Q and S188R

The normal and mutated *URO-D* alleles were expressed in *Escherichia coli* strain JM109 (Promega, Madison, WI, USA) using the pKK223-3 plasmid (Pharmacia Biotech Inc., Piscataway, NJ, USA), as previously described (20). Using three PCR reactions, missense mutations were introduced into a wild-type *URO-D* cDNA by site-directed mutagenesis (30). For each mutation, two overlapping PCR fragments containing the desired mutation were generated by PCR from normal *URO-D* cDNA and cloned into the pKK223-3 vector (pKK-URO-D).

For mutation P150Q, the two sets of primers were E5(S)/150(AS) and 150(S)/E9(AS), whereas for mutation S188R, the primer pairs were E5(S)/188(AS) and 188(S)/E9(AS) (Table 2). For both mutations, the two PCR products were used together as templates in a subsequent PCR with the external primers E5(S) and E9(AS). All reactions were performed in a final volume of 100 μl containing ×1 Tris–HCl buffer, pH 8, 1 mM of $MgCl_2$, 200 μM of each dNTP, 30 pmol of each primer, 10 ng of DNA, and 2 U of Taq polymerase (Biotools, B & M Laboratories, Madrid, Spain). PCR amplification was performed in a Perkin-Elmer thermocycler 9600 with an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Table 2. Primers used for site-directed mutagenesis

Primer	Sequence
E5(S)	5′-GGTGACCAT*GGTACC*TGGCAAAGG-3′
E9(AS)	5′-CTCAG*ATGCAT*ACAAGGCACAGG-3′
150(S)	5′-GCTGGACGTGTGCaGCTGATTGGCTTTGC-3′
150(AS)	5′-GCAAAGCCAATCAGCtGCACACGTCCAGC-3′
188(S)	5′-GAGACCTCAGGCTAGaCACCAGCTGCTTCG-3′
188(AS)	5′-CGAAGCAGCTGGTGtCTAGCCTGAGGTCTC-3′

Italicized nucleotides in primers E5(S) and E9(AS) are the restriction sites for *Kpn I* and *Nsi I*, respectively. In the mutagenesis primers, the mutated base is indicated in lower case letters. S, sense; AS, antisense.

The final PCR products were digested enzymatically with the restriction endonucleases *Kpn I* and *Nsi I* (New England Biolabs, Beverly, MA, USA). The *Kpn I* and *Nsi I* fragments were purified using the GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ) and then ligated as a cassette by exchange into the vector pKK-URO-D after digestion with the same enzymes, thus generating the mutant constructs pKK-URO-D-P150Q and pKK-URO-D-S188R.

The integrity of each expression construct was verified by automated sequencing in an ABI PRISM 310 DNA sequencer (Applied Biosystems), using the ABI PRISM dRhodamine terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). After transformation, bacterial clones either containing solely vector pKK223-3, the vector containing the normal *URO-D* cDNA, or the mutant cDNAs were grown and induced with 5 mM isopropylthiogalactoside (IPTG) for 3 h. Then, cells were harvested and washed twice in phosphate-buffered saline, and the pellet was resuspended in 250 μl of lysis buffer (250 mM potassium phosphate, pH 6.0, 0.1% Triton-X 100) and sonicated for enzyme assay.

URO-D activity was determined as previously described using pentacarboxylic acid porphyrinogen I as substrate (15). Porphyrins were converted to their methyl esters and analyzed by high-performance liquid chromatography with fluorescence detection. The specific activity was calculated as nmol of coproporphyrinogen I produced/h/mg protein.

Results

Mutations in the URO-D gene

We identified six different mutations in the *URO-D* gene, including five missense mutations and one frameshift mutation. The localization of these mutations within the *URO-D* gene, along with those mutations already identified by other groups is depicted in Fig. 1.

Three of the missense mutations detected in this study have been reported previously by other groups, whereas the other two mutations are novel ones (8,14,20). In detail, the mutations consisted of two transitions and three transversions, designated F46L, P150Q, S188R, L195F, and G281E. The causality of the two newly identified missense mutations, P150Q and S188R, in causing disease was further studied by prokaryotic

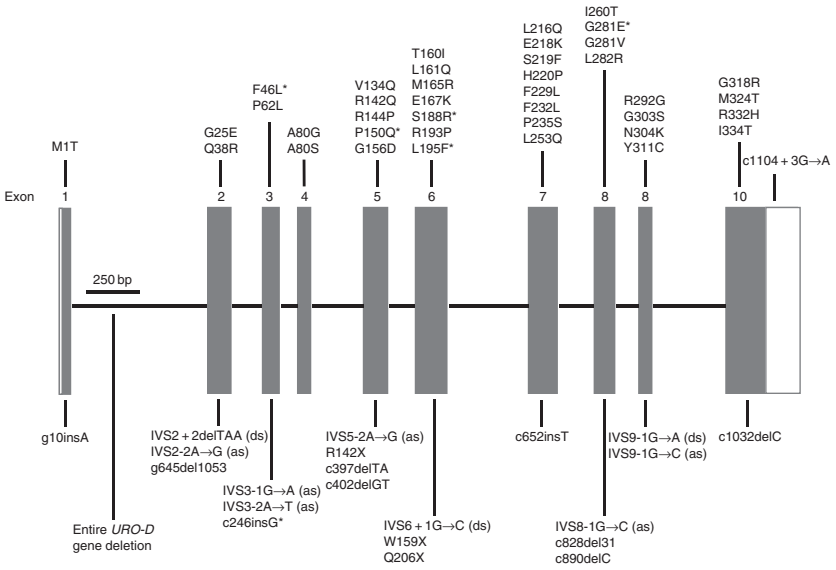

Figure 1. Schematic representation of the exon–intron organization of the uroporphyrinogen decarboxylase gene with locations of mutations reported to date. Square boxes represent exons (gray-filled boxes depicting coding sections and white-filled boxes depicting non-coding sections); black lines represent introns. Mutations marked with an asterisk were identified in this study. Nucleotides are numbered according to the published URO-D genomic sequence (20) (GenBank accession number AF047383), in which the A of the ATG initiation codon is numbered as 1. Positions at cDNA or gene level are indicated by c and g, respectively. ds, donor splice site; as, acceptor splice site.

in vitro expression. The new frameshift mutation identified in this study consisted of a single base pair insertion, designated 246insG. Of note, mutations L195F and 246insG were identified in two unrelated patients, respectively. As an illustration of the results of our mutation-detection strategy, the novel mutations identified in this study are depicted in Fig. 2.

All mutations identified in this study were initially detected by heteroduplex or complex heteroduplex formation when using CSGE analysis as mutation-screening technique. Subsequent automated sequencing of the samples displaying heteroduplexes revealed the underlying mutations. To exclude that the sequence deviations detected in this study were common polymorphisms, we studied the absence of these mutations in 200 Chilean chromosomes by a combination of heteroduplex analysis and automated sequencing (data not shown).

Decreased residual URO-D activity caused by missense mutations

To study the functional consequences of the two novel missense mutations, pKK-URO-D expression vectors for each of the mutant alleles were constructed and subsequently expressed in *Escherichia coli* to determine residual URO-D activities. As summarized in Table 3, both the P150Q and the S188R mutant alleles expressed less than 1% of wildtype URO-D activity, indicating that these mutations are most likely responsible for reduced URO-D activities in patients carrying these mutations (Table 3).

Discussion

In this study, we investigated the molecular basis of PCT in Chile by screening 18 unrelated patients for mutations in the *URO-D* gene, indicative of familial PCT. Using PCR, heteroduplex analysis, and automated sequencing, we identified six different mutations, of which three were not reported previously. Furthermore, the probability of each novel missense mutation identified in this study in causing disease was assessed by prokaryotic expression studies.

The molecular heterogeneity of type II PCT is demonstrated by different mutations reported in the *URO-D* gene to date, including missense, nonsense, frameshift, and splice site mutations (18–27) (Fig. 1).

Interestingly, in the majority of the reported cases of familial PCT, mutations are unique in each individual family. One notable exception is a frameshift mutation, designated 10insA, which was recurrently encountered in six Argentinean families (20,25). In four of them, haplotype analysis revealed that this mutation arose on three distinct backgrounds in these families, thus most likely representing a mutational hotspot in the *URO-D* gene (20). The other exception is a splice site mutation, designated IVS6+1G→C, identified in six unrelated PCT families (19,27).

Here, we also detected two recurrent mutations. Mutation L195F was encountered in two unrelated families and, additionally, mutation 246insG was identified in two other unrelated Chilean families. However, in light of the small number of families, we did not perform haplotype analyses to study putative mutational hotspots or

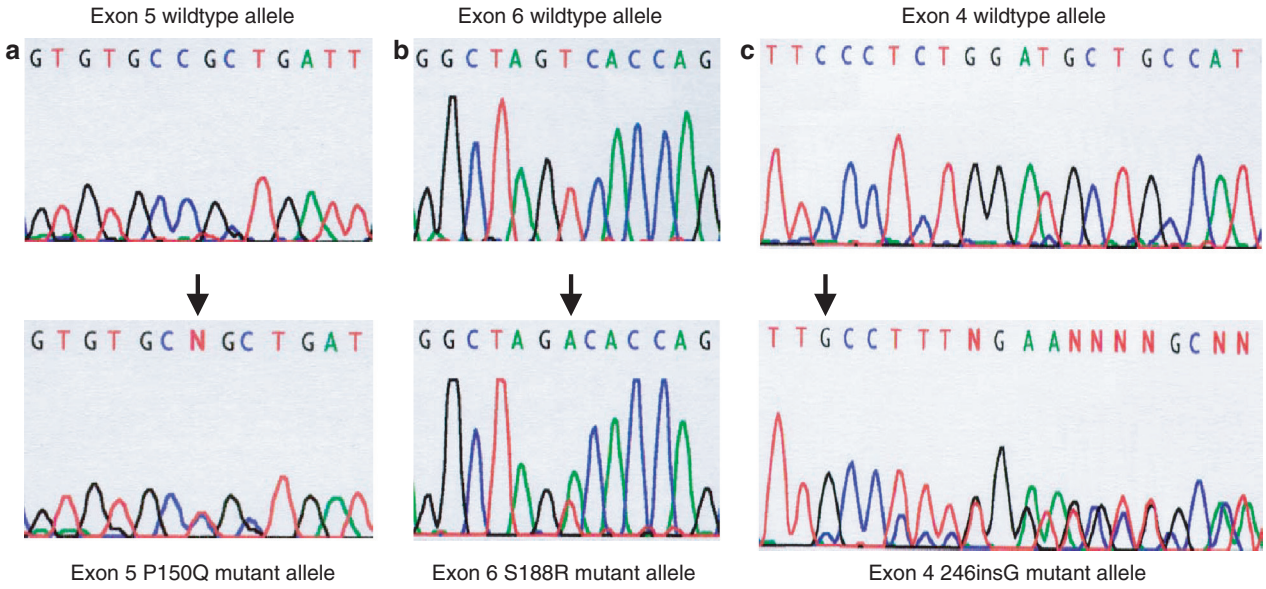

Figure 2. Results of DNA analysis in three of the 18 porphyria cutanea tarda families studied. Automated sequence analysis using the forward strand primer revealed three novel mutations in the uroporphyrinogen decarboxylase gene. The mutations consisted of two missense mutations and one frameshift mutation (a–c) as indicated by arrows in the lower panel compared with the respective wildtype sequences of a control (upper panel).

founder effects. Such studies could be useful in the near future if more PCT patients carrying one of these mutations should be detected, in particular, as L195F has now been reported in three unrelated families (20).

Interestingly, all mutations identified herein were already detected by heteroduplex formation using CSGE as screening technique. These results are comparable with those recently reported for denaturing-gradient gel electrophoresis as screening method for the detection of *URO-D* gene mutations and demonstrate the sensitivity and usefulness of CSGE in screening for *URO-D* gene defects in PCT (21).

The mutations detected in this study consisted of five missense mutations and one frameshift mutation, designated F46L, P150Q, S188R, L195F, G281E, and 246insG, respectively. Interestingly, the majority of mutations reported in the *URO-D* gene to date consist of missense mutations. This is in contrast to the predominant type of mutations encountered in another dominantly transmitted cutaneous porphyria, erythropoietic protoporphyria, where mostly premature termination codons are found, caused by frameshift or nonsense or splice site mutations (31,32).

The two novel missense mutations identified in this study markedly altered the nature of the encoded amino acids. Although the proline residue at position 150 and the serine residue at position 188 in URO-D are not invariant among different species, a comparison of the nucleotide-deduced amino acid sequences revealed both of them to be strictly conserved in human, mouse, rat and the yeast *Saccharomyces cerevisiae* through evolution. Furthermore, proline at position 150 is highly conserved in the bacteriae *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium leprae* (33). Mutation P150Q is located within a helix sheet turn between the H2 helix and the S3 strand in the N-terminal region of the $(\beta/a)_8$ barrel of the URO-D protein. This mutation leads to the substitution of a rigid neutral hydrophobic proline by a bulky polar glutamine residue, thereby probably causing a significant structural alteration. Mutation S188R occurred within the H3 helix likewise located in a barrel region of URO-D. This mutation results in an important amino acid change replacing the short, polar, and uncharged residue serine by the long positively charged residue arginine. Most importantly, as demonstrated by *in vitro*

Table 3. Prokaryotic expression of uroporphyrinogen decarboxylase (URO-D)

	URO-D activity (U/mg)		
Construct	Mean	Range	Residual activity (%)
PKK	0.40	0.37–0.42	–
pKK-URO-D	27.66	26.07–30.50	100
pKK-URO-D-P150Q	0.43	0.35–0.50	<1.0
pKK-URO-D-S188R	0.51	0.47–0.58	<1.0

Specific activities (SA) were the results of four independent experiments. Residual activity was calculated as:

$$\text{Residual activity} = 100 \times \frac{SA - SA(pKK)}{SA(pKK - URO - D) - SA(pKK)}$$

expression studies, both mutant proteins exhibit very low, if any, residual activity in a crude bacterial extract, which, in sum, indicates that these mutations have important structural and/or functional consequences in the encoded mutant URO-D enzyme.

The frameshift mutation identified in this study consisted of a G-insertion at nucleotide position 246 of the URO-D cDNA, counting the A of the initiation methionine residue as number 1. This sequence deviation results in a premature termination codon 159 base pairs downstream of the site of insertion. As premature termination codons have been shown to be strongly associated with rapid degradation of mutant transcripts by nonsense-mediated mRNA decay, no functional URO-D protein would be produced from the mutant allele (34,35).

At the moment, the Chilean population consists of approximately 15 million habitants and reflects a hybrid mixture composed of approximately 60% of European genes, 35% of Amerindian genes and 5% of genes derived from other populations. Only less than 4% of the Chilean population are born in foreign countries. Available data on approximately 50 single gene defects, among them the *URO-D* gene, indicate that the estimated prevalence of type II PCT in Chile is 1 : 50 000, thus being similar to the prevalence rates published for foreign Caucasian populations. This is also valid for the prevalence rates of autosomal dominant disorders in general, so that our data obtained in the Chilean population are comparable with those reported in other continents and countries, e.g. Europe (36,37).

Usually, the measurement of residual URO-D activity in erythrocytes was used in previous studies to distinguish patients with the sporadic form of PCT from the inherited variant (4,38–40). In this study, however, we used direct DNA analysis of the *URO-D* gene as the most stringent method to identify the inherited variant of PCT in a cohort of 18 previously unclassified PCT patients. In the current literature, the ratio between type I and type II PCT is reported to be approximately 3 : 1 to 4 : 1, depending on the respective source and population studied (1,2,4,38,40). Therefore, it is noteworthy that the ratio between familial and sporadic cases of PCT observed in this study was almost 1 : 1, with eight of 18 cases representing type II PCT. Although the total number of PCT patients studied herein is comparatively small, these 18 cases after all reflect 17.8% of the index patients with PCT registered in the Chilean Porphyria Center in Santiago (41). This center was established in 1974 and currently includes 239 index cases of porphyria, 101 of them being diagnosed with PCT (42.2%). In sum, our data strongly indicate that among Chilean PCT patients, the familial variant is more frequently encountered than in any other country in which molecular genetic studies in PCT families have been performed to date (20,23,24,26,27). The future inclusion and investigation of further Chilean individuals suffering from PCT will allow to estimate the ratio between familial and sporadic PCT more accurately.

Systematic biochemical and molecular analyses of families suffering from PCT are clinically valuable, in particular as (i) the etiopathology of type I and type III PCT is not completely understood yet and (ii) several other diseases like hemochromatosis, hepatitis C virus infection or HIV infection comprise major risk factors for this disease (42). Furthermore, strong evidence exists that individuals suffering from PCT have a significantly increased risk for the development of hepatocellular carcinoma. Thus, patients revealing severe hepatic pathology or longstanding PCT should be monitored frequently for the occurrence of hepatocellular carcinoma in the long term (43,44).

The data presented here indicate that the frequency of type II PCT is the highest one reported in any country to date and further emphasize the molecular heterogeneity encountered in this disease. The identification of mutations in the *URO-D* gene in patients and families with type II PCT has enhanced our understanding of the disorder. Once a mutation has been identified in a patient suffering from familial PCT, the detection of asymptomatic carriers within the patient's family is easily accomplished using molecular biological techniques. This will allow for early examination of these individuals for concomitant diseases frequently associated with PCT, such as hepatitis C virus infection, hemochromatosis, or hepatocellular carcinoma. Besides, our results should streamline and facilitate the elucidation of the molecular basis of PCT in Chile as well as in its neighboring countries Argentina, Peru and Bolivia. In our laboratories, further studies are underway to investigate putative genotype-to-phenotype correlations in PCT and to understand additional and unusual mechanisms of inheritance in this disease.

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